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THE EFFECTS OF INTRAVENOUS ALDOSTERONE AND HYDROCORTISONE ON THE URINARY ELECTROLYTES OF THE RECUMBENT HUMAN SUBJECT

BY J. N. MILLS, S. THOMAS AND K. S. WILLIAMSON
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(*Received* 9 *December* 1960)

The sodium retention on injection of aldosterone into human subjects is often accompanied by an acute increase in the excretion of hydrion. The changes in hydrion excretion observed by Mills, Thomas & Williamson (1960*a*) were not large, perhaps because of the rather high control outputs; we hoped that a rise in hydrion excretion might be more convincingly demonstrated in recumbent subjects, in whom urine is commonly alkaline (Thomas, 1957), and the action of aldosterone on cation excretion might be further elucidated. Injections have therefore been given to recumbent subjects either on waking in the morning, or at 13.00 hr; the effects were thus observed over two periods when the spontaneous excretion of sodium and potassium is markedly different. Since in our previous experiments hydrocortisone (cortisol) appeared to increase potassium excretion without directly affecting hydrion, we have also examined its action in the different circumstances of the present paper.

A preliminary account of this work has already appeared (Mills, Thomas & Williamson, 1960*b*).

METHODS

The subjects were three healthy males aged 27–46. In afternoon experiments the subject lay down about 11.00 hr (subjects M and T) or stood from 11.00 to 13.00 hr (subject W); all remained recumbent from about 13.00 hr, when aldosterone was given, until the end of the observations 3 or 4 hr later. DL-Aldosterone (Ciba), either the 21-monoacetate or the free alcohol, was given intravenously, either as a single dose of 500 μg, or as two doses of 250 μg with an interval of 1½ hr, or by continuous infusion at 76–106 μg/hr. The effects of these different dosages were similar. In morning experiments a single injection of 500 μg aldosterone or 100 mg cortisol (EF-Cortelan, Glaxo), was given when the subject awoke, and he remained recumbent for a further 4–5 hr. Urine was voided hourly or half-hourly and collected under paraffin, and analysed by the methods of our previous paper (Mills *et al.* 1960*a*), with the addition of chloride analysis (Sanderson, 1952). Hydrion values reported in this, as in the previous, paper were calculated as (ammonium + titratable acid phosphate − bicarbonate); in most experiments titratable acid has been directly determined, and if this is substituted for titratable acid phosphate a higher figure for hydrion is obtained. Our conclusions concerning the alterations in hydrion excretion affected by steroids are, however, the same.

In afternoon experiments the subject had a light breakfast without caffeine, and no food thereafter. In morning experiments T had a similar light breakfast, but M and W fasted. The subjects drank freely.

RESULTS

Afternoon experiments

Urine collections have been made without (*N*) and with (*A*) aldosterone injection, upon three subjects, as follows: M, (4*N* and 3*A*), T (2*N* and 2*A*) and W (3*N* and 2*A*). The differences between the means of experiments with and without aldosterone injection are shown in Table 1. The differences before injection represent random differences between one day and another.

TABLE 1. Effect of aldosterone injection into recumbent subjects at 13.00 hr. Figures are differences (μequiv/min) between the means of experiments with and without aldosterone injection. Positive values indicate increased excretion

Subject		Before injection	1st hr	2nd hr	3rd hr	4th hr
M	Na	−32	+67	−16	−30	−108
	K	−16	+39	+35	+36	+36
	H	+5	−16	+5	+21	+55
	Total cation	−43	+90	+24	+27	−17
	Cl	−47	+68	+3	−37	−10
T	Na	−12	−36	−93	−84	−61
	K	−6	−18	+18	+16	+25
	H	−1	+21	+20	+20	+22
	Total cation	−19	−33	−55	−48	−14
	Cl	+17	−5	−52	−38	−15
W	Na	−22	−5	+24	−50	−122
	K	−17	−17	+14	+23	+29
	H	−2	+4	+21	+24	+30
	Total cation	−37	−18	+59	−3	−63
	Cl	−32	−1	+69	−10	−50

Every injection of aldosterone was followed by the familiar sharp fall in sodium excretion to values usually below 100 μ-equiv/min; in all control experiments it remained fairly high, usually around 200 μ-equiv/min. Potassium excretion, in the two subjects who lay down at 11.00 hr, fell continuously throughout the afternoon; after aldosterone injection the fall was smaller. In subject W, who always stood from 11.00 to 13.00 hr, excretion had fallen by 13.00 hr to 30–40 μ-equiv/min, and when he lay down without aldosterone injection it stayed around this level or rose moderately; when aldosterone was injected at the time he lay down, it rose sharply to 75–90 μ-equiv/min between 16.00 and 17.00 hr. Thus in all subjects potassium excretion was greater after aldosterone injection than in controls. This action of aldosterone upon sodium and potassium excretion has been frequently reported; it resulted in the familiar gross depression of urinary Na:K ratio.

Less familiar is the action upon acid output. In all subjects the hydrion excretion after aldosterone injection was substantially greater than in control experiments. Subjects M and T, while recumbent between 11.00 and 13.00 hr, usually excreted more bicarbonate than ammonium + titratable acid, that is, the hydrion excretion was negative; on continued recumbency hydrion output rose steadily in controls, and more steeply after aldosterone injection. In subject W, who stood from 11.00 to 13.00 hr, hydrion output was positive over this period, and fell during recumbency from 13.00 to 17.00 hr; aldosterone injection reversed this change, and led to a rise of hydrion output during sustained recumbency.

Since the outputs in different subjects were not identical, a numerical value for the mean effect of aldosterone would have little meaning. Where, however, the direction of change in excretion induced by aldosterone was the same in all three subjects, the significance of this change can be assessed by calculating P values for each subject and combining them by the χ^2 method of Fisher (1941). Only in the fourth hour after injection were the fall in sodium and the rise in potassium and hydrion highly significant ($P < 0{\cdot}01$). In the third hour the rise of potassium was just ($0{\cdot}05 > P > 0{\cdot}02$) and of hydrion not quite ($0{\cdot}10 > P > 0{\cdot}05$) significant.

Where the fall in sodium excretion was not balanced by a rise in the excretion of potassium and hydrion, chloride excretion fell, suggesting a reabsorption of some sodium in association with chloride.

Morning experiments: aldosterone and cortisol

Urine was collected from each of the subjects on two mornings without injection and on two mornings after injection, on waking, of each steroid. The electrolyte outputs are shown in Fig. 1. The mean outputs after cortisol and in control experiments are plotted, for clarity, but aldosterone experiments are plotted separately to indicate the differences between the two.

In the control experiments excretion of sodium, chloride and potassium rose to fairly high levels by the end of the morning, and hydrion excretion was negative. Aldosterone checked the rise of sodium excretion in all subjects: in T and W it checked the fall of hydrion without affecting potassium excretion; in M it increased potassium, rather than hydrion, excretion. In one experiment each on T and W the sodium retention induced by aldosterone was roughly equal to the extra excretion of potassium + hydrion, whilst in the other, and in both on M, it was obviously greater, and chloride excretion was reduced.

After cortisol injection sodium excretion behaved in much the same way as after aldosterone, except that in M the fall in excretion was delayed. The potassium excretion rose to very high levels, but hydrion excretion

was similar to or below that in control experiments. Chloride excretion appears to have decreased in subject M.

A single injection of cortisol given to subject T in the afternoon led to closely similar alterations in electrolyte excretion; the mean values are shown in Table 3.

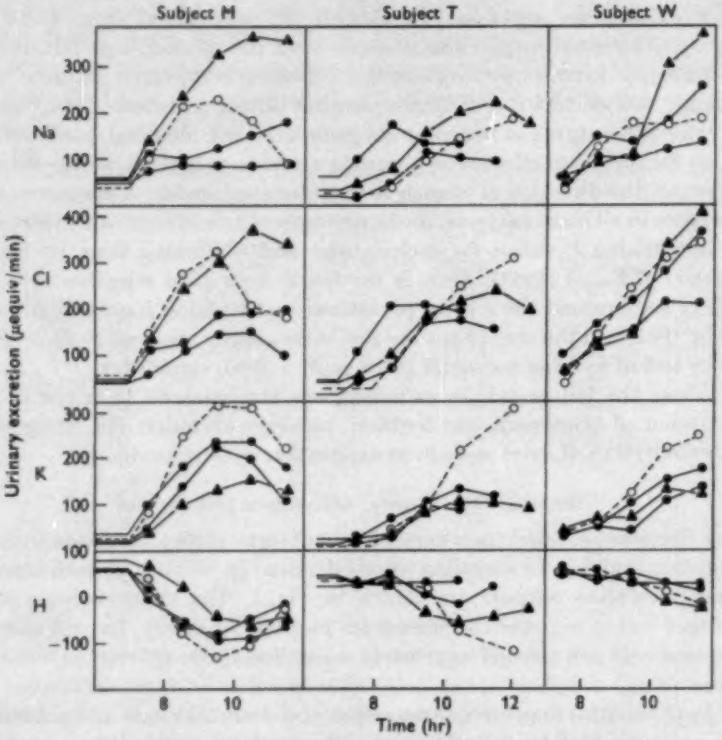

Fig. 1. Morning experiments, steroid injection on waking, about 07.00–08.00 hr. Excretion of chloride, sodium, potassium and hydrion during each succeeding hour ▲——▲, mean of two experiments without injection. ○- - - -○, mean of two experiments with cortisol injection. ●——●, individual experiments with aldosterone injection. Initial values for subjects M and T represent mean excretion during the night, subsequent points for all subjects are plotted at the mid times of collection periods.

Creatinine excretion

Creatinine excretion was measured in all experiments. There were no regular nor significant differences between the outputs in experiments without or with aldosterone or cortisol injection (Table 2), nor did exami-

nation of the output in successive hours after either steroid suggest that creatinine excretion was altered by either.

TABLE 2. Mean creatinine excretion between 13.00 and 17.00 hr in afternoon experiments, and between 08.00 (subject T) or 07.00 (subjects M and W) and 12.00 hr in morning experiments

Subject	Time	Creatinine excretion (mg/min)		
		Control	Aldosterone	Cortisol
M	Afternoon	1·18	1·27	—
T	Afternoon	1·06	1·10	1·13
W	Afternoon	1·22	1·26	—
M	Morning	1·22	1·40	1·37
T	Morning	1·07	1·17	1·18
W	Morning	1·46	1·42	1·29

DISCUSSION

The present paper extends our findings on the actions of aldosterone and cortisol to control backgrounds where excretion of sodium, potassium and chloride is high, and of hydrion negative. The mean results of the present and previous paper are summarized in Table 3. An increased excretion of hydrion was a more notable consequence of aldosterone injection when the acid output was spontaneously low, in the recumbent subject. Aldosterone was also capable of raising potassium excretion above the high level characteristic of the morning (subject M). It also appears that in the recumbent subject, when the sodium excretion is very high, the sodium retention induced by aldosterone may be much greater than the extra excretion of other cations. In these circumstances chloride excretion falls in parallel with that of sodium. Sodium retention thus always follows aldosterone injection; but it is to some extent unpredictable whether this will be associated with extra excretion of potassium or of hydrion, or diminished excretion of chloride.

The differences between the effects of cortisol and aldosterone which we previously suggested have been amply confirmed. In the doses used cortisol caused a similar or rather smaller sodium retention, but the high excretion of potassium after cortisol injection was far above the levels ever induced by aldosterone. In addition, cortisol differed from aldosterone in that it never increased, and commonly decreased, hydrion excretion.

The literature upon acute effects of aldosterone and cortisol injection has been discussed in our earlier paper (Mills *et al.* 1960*a*). No workers report full calculation of hydrion output, but in other respects published reports are in conformity with our own. When sodium excretion is large, aldosterone usually reduces it by an amount greater than the increase in potassium excretion (Ross & Bethune, 1959; Ross, Reddy, Rivera & Thorn, 1959); and a diminished chloride excretion, similar to that which

TABLE 3. Acute effects of aldosterone (0·5 mg) and cortisol (100 mg) upon electrolyte outputs in normal subjects. At night, aldosterone was injected intramuscularly; all other injections were intravenous. In some afternoon experiments aldosterone was given in a divided dose, or by continuous intravenous infusion. The outputs tabulated, in μequiv/min, are the whole output during a night's sleep, or the mean output over the period of daytime observation excluding the first hour after injection. *N* = control, *A* = aldosterone, *C* = cortisol

		Na			K			H			Cl		
		N	*A*	*C*	*N*	*A*	*C*	*N*	*A*	*C*	*N*	*A*	*C*
Seated, injection 13.00 hr. (Mills, Thomas & Williamson, 1960*a*)	A	76	7	40	36	63	130	−3	18	−54	68	39	84
	F	134	84	78	49	85	130	29	−1	−13	122	99	161
	K	95	21	38	18	30	84	35	55	20	86	46	84
	M	133	18	27	58	83	67	34	53	17	125	58	62
	T	40	10	13	22	52	67	16	33	12	40	32	48
Night, sleeping; injection at bedtime. (Mills *et al.* 1960*a*)	H	79	34	30	21	24	76	47	62	34	58	35	58
	F	75	36		24	38		35	41		64	43	
	I	114	94	114	38	45	98	40	30	34	99	117	152
	T	34	16	24	14	22	26	38	44	25	31	25	38
	W	37	28	38	26	33	55	53	65	48	32	41	70
Morning, recumbent; injection on waking. (Present paper)	M	322	113	178	126	190	272	−33	−67	−90	314	149	270
	T	199	116	146	97	107	212	−35	14	−76	218	183	248
	W	342	172	178	149	140	233	−16	23	−3	371	269	326
Recumbent, injection 13.00 hr. (Present paper)	M	114	80		35	75		14	42		146	120	
	T	98	25	12	26	45	92	16	37	0	80	53	67
	W	215	127		56	82		28	54		220	193	

we observed, has been reported in one subject by Muller, Mach & Naegeli (1955). A large excretion of potassium after cortisol injection has been reported by Raisz, McNeely, Saxon & Rosenbaum (1957) in two subjects, and by Dingman, Finkenstaedt, Laidlaw, Renold, Jenkins, Merrill & Thorn (1958) in three subjects.

There are four principal means, known or proposed, whereby sodium excretion may diminish: a fall in the amount filtered, usually due to diminishing filtered volume; increased reabsorption in association with anion, largely chloride; ion exchange for potassium in the distal tubule (Berliner, Kennedy & Hilton, 1950) or in the collecting ducts (Sullivan, Wilde & Malvin, 1960); and ion exchange for hydrion, in the distal tubule (Pitts & Alexander, 1945) or in the collecting ducts (Ullrich, 1959; Sullivan *et al.* 1960). Cortisol often increases G.F.R. (Raisz *et al.* 1957; Mills & Thomas, 1958; Mills *et al.* 1960*a*). This is probably the cause of the occasional reported rise of sodium and chloride excretion, as suggested by Laidlaw, Dingman, Arons, Finkenstaedt & Thorn (1955), whilst the more usual fall in excretion must be due to increased tubular reabsorption. We have been unable to find, either in our previous experiments (Mills *et al.* 1960*a*), in the creatinine outputs here reported, or in the work of others, any evidence that aldosterone has any consistent effect upon G.F.R.; it therefore probably lowers sodium excretion by increased tubular reabsorption.

The figures assembled in Table 3 support the hypothesis tentatively advanced in our earlier paper, that aldosterone exerts its primary effect upon a variety of mechanisms of sodium reabsorption, since the common finding in all the circumstances tabulated is the sodium retention. Two principal processes may be involved (Pitts, 1952). When sodium output is large, it seems that the extra reabsorption is mainly in association with chloride. This is known to operate in the proximal tubule, where sodium is actively reabsorbed (Oken, Whittembury, Windhager, Schatzmann & Solomon, 1959) and chloride follows passively (Giebisch & Windhager, 1959). The same may operate further down the nephron, and there is at present no evidence enabling us to locate, within the nephron, the action of aldosterone on sodium reabsorption in association with anion. The second reabsorptive mechanism, ion exchange of sodium for other cation in the distal tubule or collecting duct, appears to be the only one affected by aldosterone when sodium excretion is low, as at night; it is however also involved in the action of aldosterone when sodium excretion is high. Edmonds & Wilson (1960) produced evidence that fludrocortisone, which is said to act in the same manner as aldosterone, also promotes sodium reabsorption by these two distinct mechanisms. We cannot profitably speculate what directs the ion exchange towards hydrion or potassium

in preference, although it is not surprising that hydrion usually plays a major part in the ion exchange process when its excretion is initially low, and a minor part when its excretion is initially high.

In marked contrast to aldosterone, cortisol seems to stimulate primarily the Na $\rightleftharpoons$ K exchange, and the Na $\rightleftharpoons$ H exchange may suffer by competition. Urines with a pH of 7·5 and 7·6 have been produced on two occasions after cortisol injection. Such urinary alkalinity strengthens the argument of our previous paper (Mills *et al.* 1960*a*), that the low hydrion excretion after cortisol is not only due to lack of urinary buffer.

Further techniques are necessary to establish which ion transport mechanisms are primarily affected by steroids. It appears, however, that Na $\rightleftharpoons$ K exchange is specifically stimulated by cortisol, whereas aldosterone stimulates all three suggested mechanisms of sodium reabsorption, in association with chloride, in exchange for potassium and in exchange for hydrion.

SUMMARY

1. Electrolyte excretion has been measured in subjects recumbent either from waking in the morning until midday, or from 13.00 to 17.00 hr, with or without intravenous injection of aldosterone or cortisol.

2. Sodium, and usually chloride, excretion were greatly reduced by aldosterone, in the morning and the afternoon.

3. Potassium excretion was moderately increased by aldosterone in the afternoon, and in one of three subjects in the morning.

4. Hydrion excretion, which was low in control experiments at both times of day, usually rose considerably after aldosterone injection.

5. After cortisol injection potassium excretion was consistently increased, sodium and hydrion excretion less consistently diminished, and chloride excretion little affected.

6. The observations accord with the hypothesis that aldosterone exerts its primary action upon reabsorption of sodium either in association with anion such as chloride, or by cation exchange with potassium or hydrion, or by a combination of these mechanisms; cortisol, by contrast, appears to promote mainly the Na $\rightleftharpoons$ K exchange mechanism.

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REFERENCES

Berliner, R. W., Kennedy, T. J. & Hilton, J. G. (1950). Renal mechanisms for excretion of potassium. *Amer. J. Physiol.* **162**, 348–367.

Dingman, J. F., Finkenstaedt, J. T., Laidlaw, J. C., Renold, A. E., Jenkins, D., Merrill, J. P. & Thorn, G. W. (1958). Influence of intravenously administered adrenal steroids on sodium and water excretion in normal and Addisonian subjects. *Metabolism*. **7**, 608–623.

EDMONDS, C. J. & WILSON, G. M. (1960). The action of hydroflumethiazide in relation to adrenal steroids and potassium loss. *Lancet*, **278**, 505–509.

FISHER, R. A. (1941). *Statistical Methods for Research Workers*, 8th ed., pp. 97–99. Edinburgh: Oliver and Boyd.

GIEBISCH, G. & WINDHAGER, E. E. (1959). Chloride fluxes across single proximal tubules of necturus kidney. *Fed. Proc.* **18**, 52.

LAIDLAW, J. C., DINGMAN, J. F. ARONS, W. C., FINKENSTAEDT, J. T. & THORN, G. W. (1955). Comparison of the metabolic effects of cortisone and hydrocortisone in man. *Ann. N.Y. Acad. Sci.* **61**, 315–323.

MILLS, J. N. & THOMAS, S. (1958). The acute effects of cortisone and cortisol upon renal function in man. *J. Endocrinol.* **17**, 41–53.

MILLS, J. N., THOMAS, S. & WILLIAMSON, K. S. (1960*a*). The acute effect of hydro cortisone deoxycorticosterone and aldosterone upon the excretion of sodium, potassium and acid by the human kidney. *J. Physiol.* **151**, 312–331.

MILLS, J. N., THOMAS, S. & WILLIAMSON, K. S. (1960*b*). The different effects of aldosterone and cortisol in recumbency. *J. Physiol.* **153**, 56*P*.

MULLER, A. F., MACH, E. & NAEGELI, H. (1955). Comparison of the effect of aldosterone and cortexone (desoxycorticosterone) on electrolyte excretion in man. *Acta endocr., Copenhagen*, **20**, 113–130.

OKEN, D. E., WHITTEMBURY, G., WINDHAGER, E. E., SCHATZMANN, H. J. & SOLOMON, A. K. (1959). Active sodium transport by the proximal tubule of *Necturus*. *J. clin. Invest.* **38**, 1029.

PITTS, R. F. (1952). Effects of adrenal cortical hormones on renal function. In RALLI, E. P. *Adrenal Cortex*, Trans. of the Third Conference, pp. 11–53. New York: Josiah Macy Foundation.

PITTS, R. F. & ALEXANDER, R. S. (1945). The nature of the renal tubular mechanism for acidifying the urine. *Amer. J. Physiol.* **144**, 239–254.

RAISZ, L. G., MCNEELY, W. F., SAXON, L. & ROSENBAUM, J. D. (1957). The effects of cortisone and hydrocortisone on water diuresis and renal function in man. *J. clin. Invest.* **36**, 767–779.

ROSS, E. J. & BETHUNE, J. E. (1959). Antagonism between the effect of aldosterone and a synthetic steroid lactone on the renal excretion of sodium and potassium in man. *Lancet*, **276**, 127–129.

ROSS, E. J., REDDY, W. J., RIVERA, A. & THORN, G. W. (1959). Effects of intravenous infusions of DL-aldosterone acetate on sodium and potassium excretion in man. *J. clin. Endocrin.* **19**, 289–296.

SANDERSON, P. H. (1952). Potentiometric determination of chloride in biological fluids. *Biochem. J.* **52**, 502–505.

SULLIVAN, L. P., WILDE, W. S. & MALVIN, R. L. (1960). Renal transport sites for K, H and NH_3. Effects of impermeant anions upon their transport. *Amer. J. Physiol.* **198**, 244–254.

THOMAS, S. (1957). Some effects of change of posture on water and electrolyte excretion by the human kidney. *J. Physiol.* **139**, 337–352.

ULLRICH, K. J. (1959). Das Nierenmark, Struktur, Stoffwechsel und Funktion. *Ergebn. Physiol.* **50**, 433–489.

J. Physiol. (1961), **156**, *pp.* 424–444
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THE RELEASE OF ADRENALINE AND NORADRENALINE FROM THE ADRENAL GLANDS OF THE FOETAL SHEEP

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The adrenal medulla of some foetal and new-born animals contains a disproportionately large amount of noradrenaline when compared with that of the adult (Shepherd & West, 1951, Hökfelt, 1952). This difference does not occur invariably and may be related to the degree of development of the sympathetic system in different species. Thus, in the rabbit, which has a high noradrenaline content in the adrenal medulla at birth, autonomic nervous control of the circulation is not developed at this stage (Bauer, 1938; Dawes, Handler & Mott, 1957). A similar relationship between a relatively inefficient sympathetic system and a high medullary noradrenaline content appears to be present in the calf (Holton, 1951; Comline & Titchen, 1951; Silver, 1954). In the lamb, on the other hand, maturation of the sympathetic system, as gauged by the gradual increase in the magnitude of the foetal blood pressure and its response to asphyxia, appears to occur during the latter part of foetal development and not after birth (Born, Dawes & Mott, 1956).

These observations do not, however, provide any information on the potential activity of the foetal adrenal medulla, nor do they give any direct evidence on whether a high concentration of noradrenaline within the glands of foetal and new-born animals can in fact be released by a stimulus such as asphyxia. Indeed, Silver (1960) found that in the young calf the proportions of adrenaline and noradrenaline released into the adrenal effluent blood by splanchnic nerve stimulation did not conform in any consistent manner to the percentage of either amine stored in the glands.

In the experiments reported here, the concentrations of adrenaline and noradrenaline in the adrenal effluent blood of the sheep foetus have been estimated and the output during asphyxia compared with that during stimulation of the splanchnic nerves; the results have also been compared with those obtained in the lamb and in the adult. A short account of the preliminary experiments has been published previously (Comline & Silver, 1958).

METHODS

Animals

Welsh Mountain and Border Leicester sheep and lambs were used in these experiments. In the pregnant animals the date of service was known, and foetuses were used from the 80th day of gestation until term (147 ± 3 days). An additional check on foetal age was afforded by measurements of foetal body weight and adrenal weight.

Preparation

Lambs and adult sheep. Anaesthesia was induced and maintained by the intravenous injection of sodium pentobarbitone (Nembutal, Abbott Laboratories). Effluent blood was collected from the left adrenal gland, which was exposed by an incision through the abdominal wall parallel to the last rib; thereafter the preparation, cannulation and collection of the adrenal effluent blood was similar to that described for the calf (Silver, 1960). Between samplings the adrenal venous blood was returned through a Murphy drip chamber connected to the cannula in the femoral vein.

Foetal lambs. The uterus was exposed by a mid line abdominal incision along the linea alba. The foetus was removed from it and placed on a palette-shaped board immediately above the abdomen. With this procedure the umbilical cord lay in the cleft of the palette and was thereby protected from stretch or trauma during the subsequent dissection and experimental procedures. The foetus was covered with thick gauze pads to avoid undue heat loss. Throughout the experiment a mixture of 95 % O_2 and 5 % CO_2 was given to the ewe at the rate of 2 l./min through a side tube in the tracheal cannula. A mask was placed over the nose of the foetus immediately after removal from the uterus and a clamped cannula filled with warm NaCl solution (0·9 g/100 ml.) was inserted into the trachea as quickly as possible to prevent any effective respiration. When required, intravenous injections were given to the foetus through a cannula in the jugular vein; blood pressure was recorded with a small bore mercury manometer from the carotid artery. Coagulation of the blood was prevented by the intravenous injection of heparin (1000 i.u./kg).

The method of collecting venous blood from the left adrenal gland was essentially similar to that in the adult. After removal of the left kidney, the adrenal effluent blood was diverted backwards into the cannulated left renal vein by curved forceps clamped across the junction of the renal vein with the vena cava. A return circuit for the adrenal effluent blood to the general circulation was not practicable in the foetus; the forceps were therefore placed in position just before the collection of adrenal venous blood and removed immediately afterwards. This method allowed several samples to be taken without undue blood loss.

Splanchnic nerves. Ligatures were placed round the two main branches of the left splanchnic nerve before they were cut close to the point at which they emerged from under the diaphragm. In those experiments in which complete denervation of the adrenal was required, any other branches of the splanchnic plexus which appeared to supply the adrenal gland were cauterized, and at the end of the experiment the area was carefully examined for any small fibres of the splanchnic nerves which remained intact.

Stimulation of the peripheral ends of the left splanchnic nerves was carried out by means of a Palmer induction coil (frequency *ca.* 80/sec). The stimulus strength was always adjusted to give a maximal increase in blood pressure. In foetal lambs between 80 and 105 days the splanchnic nerves were very short and fragile and therefore the left adrenal gland was stimulated directly by placing the electrodes across its dorsal and ventral surfaces. At 115 days both methods of stimulation were used.

Hexamethonium ('Vegolysen'; May & Baker) was injected intravenously into foetal lambs only, at a dose of 10 mg/kg and, when possible, the degree of transmission failure in the adrenal medulla was checked by measuring pressor amine output during splanchnic nerve stimulation before and after its administration.

Pithing and spinal cord transection in foetal lambs. Pithing was carried out after removal of the dorsal arches of the last two or three lumbar vertebrae to allow the insertion of a probe which was passed up the centre of the spinal cord until flexion of the forelimbs ceased; this was taken to indicate the destruction of the spinal cord at least to the level of the sixth cervical vertebra.

Transection of the spinal cord was carried out in the atlantoccipital space in foetal and new-born lambs. The cord was exposed and a loose ligature placed round it, care being taken to avoid damage to the venous sinuses during this procedure. Subsequent tying of the ligature and section of the cord was carried out about 10 min before subjecting the foetus to asphyxia. The efficacy of all these procedures was checked at the end of the experiment.

Asphyxia. In the foetus this was induced by ligation of the umbilical cord. The onset of asphyxia was rapid, and samples were collected 15 sec–1 min later according to the rate of blood flow through the gland.

In the adult sheep and young lambs asphyxia was induced by allowing the animal to rebreathe from a balloon filled with N_2 attached to the end of the tracheal cannula. Since these animals were used for comperison with the foetus no attempt was made to separate the effects of anoxia from those of hypercapnia. The blood samples were collected from 2 min after the onset of asphyxia for a further 2–3 min, depending on the degree of circulatory failure. In the majority of experiments the animal was revived with O_2 until the blood pressure returned to normal. A further period of at least 20 min without additional O_2 was allowed before repetition of the asphyxia.

Adrenal glands. In lambs and non-pregnant adult sheep the adrenal glands were removed at the end of the experiment, i.e. about 1–2 hr after the onset of anaesthesia. The right adrenal glands were used for the estimation of adrenaline and noradrenaline, and were removed only from animals which had been used for stimulation of the left adrenal gland by the splanchnic nerves; glands were not used from animals which had been subjected to or allowed to die from prolonged asphyxia.

Estimations of pressor amine content were made in two series of adrenal glands from foetal lambs and pregnant ewes of the Welsh breed. In series 1, during the 1956 breeding season, the foetal adrenal glands were removed after death from asphyxia, since no other animals were available; the right maternal glands were taken about 2 hr later, after an experiment involving stimulation of the left splanchnic nerve, i.e. 3–4 hr from the induction of anaesthesia. In series 2, twelve foetuses aged from 80 to 144 days were used during 1959 and 1960 solely for the collection of foetal adrenal glands for the estimation of their adrenaline and noradrenaline content. In these animals both adrenal glands were removed with the minimum degree of disturbance or operative trauma within 30–50 min from induction of anaesthesia in the ewe; adrenalectomy in the ewe was performed immediately afterwards. All adrenals were weighed and immediately ground with sand and 0·1 N-HCl (10 ml. acid/g whole adrenal).

Adrenal effluent blood samples. In the lambs and adult sheep 10–15 ml. blood was usually collected in 2–3 min according to the rate of blood flow; in any one animal the sampling period was kept constant. In the foetal lambs the rate of blood flow from the adrenal gland was slow, and the amount collected and the sampling time varied with age. Where possible 3–5 ml. was collected over a 5 min period, but in the 80–100 day animals only 1–3 ml. was generally obtained after 10–15 min. Between 105 and 125 days 5 ml. blood could usually be collected within 5–10 min, and thereafter a 5 min sampling period was employed. The blood flow increased during asphyxia, and under these conditions the sampling period for 80–105 day foetuses was 10 min; with the older foetuses it could be reduced to a 5 min. period, and after birth 2 min was allowed for the collection of each sample.

Polythene or nylon tubing and siliconed glassware were used throughout; the blood was collected in ice-cold vessels and immediately centrifuged on ice. Samples collected from lambs and adult sheep yielded 3–5 ml. plasma for pressor amine extraction, while the amount of plasma available from the foetal blood samples varied from 0·25 to 2·5 ml.

Adrenaline and noradrenaline estimation. The method of extraction of the plasma and adrenal tissue was the same as that described previously (Silver, 1960), with the chromatographic procedure developed by Crawford & Outschoorn (1951) and Vogt (1952) for the separation of the amines. They were then estimated either by biological assay, from the blood pressure response of the anaesthetized rat (Outschoorn, 1952), or by fluorimetry by the method of von Euler & Floding (1955). Good agreement between these two methods has already been reported (Silver, 1960). Owing to the low pressor amine content of some of the foetal adrenal blood samples, more stringent precautions had to be taken to reduce extraneous fluorescence in the plasma. Blank fluorescence of control plasma samples (containing no adrenaline or noradrenaline) was reduced by using freshly distilled phenol for chromatography and redistilled A. R. quality benzene for washing the chromatograms free from all trace of phenol, and by omitting ascorbic acid from the eluting fluid (NaH_2PO_4 0·4 g/100 ml.). It was found unnecessary to concentrate the eluates by evaporation for estimation by fluorimetry; the samples were therefore made up to a standard volume and those which contained more than 0·5 μg of either amine were stored at $-20°$ C for periods varying from a few days to 2–3 weeks; no losses were detected during this period. Samples containing smaller quantities of amine were estimated as soon as possible after elution, since storage for more than a few days sometimes resulted in loss of activity. The effect of control plasma eluates on the development of fluorescence in standard adrenaline and noradrenaline solutions was always checked. The amount of adrenaline and noradrenaline in all samples are expressed as the base and not as the salt.

RESULTS

Adrenaline and noradrenaline in the adrenal glands

Foetal lambs. A rise in the percentage of adrenaline in the adrenal glands of the lamb during foetal development has already been reported (Comline & Silver, 1958). This rise was due to a marked increase in the total amount of adrenaline in the glands during late pregnancy with little change in noradrenaline content. In these experiments adrenal glands for estimation were taken from animals which had been subjected to prolonged asphyxia, since no other material was available. Wide variations in content were encountered, and in view of the marked pressor amine discharge, particularly of noradrenaline, which occurs under these conditions (Comline & Silver, 1958) as well as the possibility of a slow depletion during anaesthesia (Elmes & Jefferson, 1942), a further series of animals were used in which interference in any form was kept to a minimum. When the results of both sets of experiments are compared (Table 1) it can be seen that the foetal adrenal glands removed after minimum trauma contained approximately twice as much adrenaline and three times as much noradrenaline per gram of tissue as the corresponding glands removed after asphyxia; consequently the percentage of adrenaline no longer increased with age, but declined from 80 days until 120 days, and subsequently rose and reached 58 % near term.

The total amounts of adrenaline and noradrenaline contained in the glands also differed considerably in the two series (Fig. 1); after asphyxia

(*a*) the amounts of both amines were considerably lower at all ages than in the animals in which interference was kept to a minimum (*b*). Furthermore, in the latter there was a rapid increase in the noradrenaline content between 80 and 110 days which was barely evident after asphyxia. On the other hand, the major rise in adrenaline content occurred towards the end of pregnancy in both groups of animals.

TABLE 1. The concentration of adrenaline (Ad) and noradrenaline (NAd) in foetal adrenal glands removed either after a minimum period of anaesthesia or after death of the foetus from asphyxia

	After asphyxia				After minimum anaesthesia			
Foetal age (days)	No. of animals	Mean concn. (mg/g adrenal) Ad	NAd	Mean % adrenaline	No. of animals	Mean concn. (mg/g adrenal) Ad	NAd	Mean % adrenaline
80–90	4	0·22	0·27	45	2	0·55	0·41	52
100–105	4	0·28	0·30	48	3	0·83	0·90	47
116–124	2	0·36	0·32	53	2	0·70	1·03	42
132–137	3	0·56	0·33	63	3	1·0	1·03	49
142–144	2	0·66	0·28	70	2	0·85	0·67	58

The values for the pressor amine content of glands from animals of the same age were by no means uniform, even when stringent precautions were taken to keep the conditions for adrenalectomy constant. However, the content of the right and left adrenal glands in any one animal was always similar and the differences between animals could only be ascribed to individual variations. Throughout pregnancy no relation could be found between the weights of the foetal adrenal glands and the amounts of either adrenaline or noradrenaline contained within them. Thus, at term, the abrupt increase in the weight of the foetal glands (Fig. 1) was not accompanied by any comparable changes in content; it was, therefore, ascribed to growth of the adrenal cortex rather than the medulla.

Lambs and adult sheep. In the lambs and non-pregnant sheep the values for pressor amine concentration, given in Table 2, were obtained from the right adrenal glands removed 1–2 hr after the induction of anaesthesia in experiments during which the left splanchnic nerve and adrenal glands were stimulated; consequently some depletion of the right adrenal glands may have occurred and the figures are not, therefore, entirely comparable with those from either series of foetuses. Although the total amounts of both amines increased with age, no marked changes were found either in their concentrations or in the percentage of adrenaline after birth, and in this species it is clear that the major changes occur in the foetus.

In the pregnant animals the conditions of the experiments were different in the two groups. In series 1 the right adrenal gland was removed for assay 3–4 hr after induction of anaesthesia; during this time the foetus

had been removed and subsequently the left maternal adrenal gland stimulated through the splanchnic nerve. In these preparations the concentration of both pressor amines was very low. In series 2, however, the

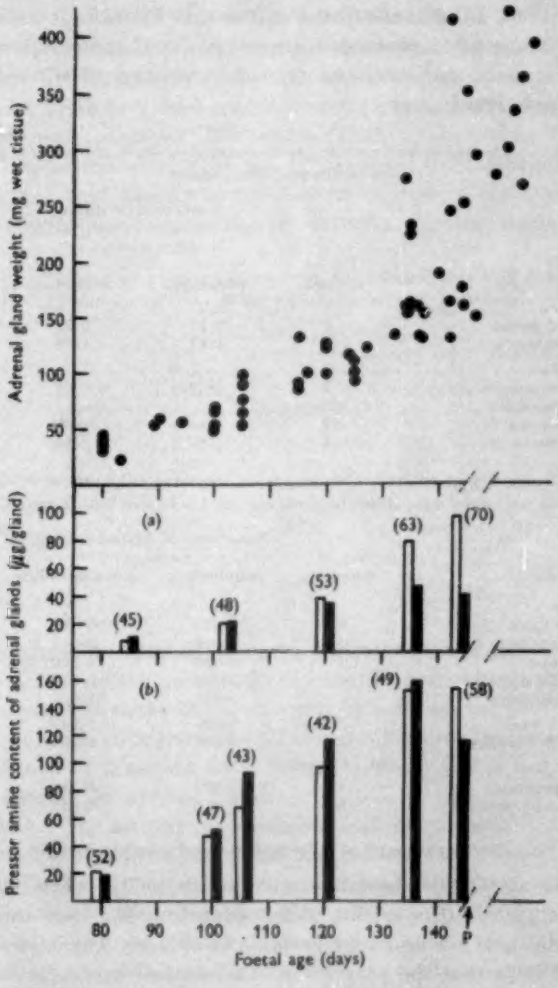

Fig. 1. The change in weight (above) and total pressor amine content (below) of the foetal adrenal glands of the lamb with age: ■, noradrenaline; □, adrenaline; % adrenaline in parentheses above each histogram. Adrenals removed (*a*) after death of lambs from asphyxia, (*b*) after minimum duration of anaesthesia. P, parturition.

maternal adrenal glands were removed after 40–60 min of anaesthesia, i.e. immediately after foetal adrenalectomy. The differences between the adrenaline and noradrenaline concentrations in the two groups after the different experimental treatments (Table 2) resemble those found in the foetus (Table 1). In both the foetus and the ewe the noradrenaline content was much lower after prolonged anaesthesia and more rigorous experimental treatment, and consequently the percentage of adrenaline in the adrenal glands was higher.

TABLE 2. Adrenaline and noradrenaline concentration in the adrenal glands of lambs and adult sheep

Age	No. of animals	Mean pressor amine concentration (mg/g whole adrenal tissue)		Mean % adrenaline
		Adrenaline	Noradrenaline	
Lambs				
0–2 weeks	4	0·47	0·23	67
7–8 weeks	4	0·61	0·28	69
Adults				
Non-pregnant	4	0·72	0·29	71
Pregnant				
Series 1	16	0·46	0·08	85
Series 2	6	0·87	0·29	75

TABLE 3. Resting output of pressor amines from the adrenal glands of sheep during pre- and post-natal life; splanchnic nerves cut 15–30 min before sampling

Age	No. of animals	Mean rate of output (μg/min)		Mean % adrenaline
		Adrenaline	Noradrenaline	
Foetus				
80–90 days	8	0·025	0·033	43
100–105 days	6	0·019	0·017	53
115 days	2	0·026	0·040	40
126–134 days	4	0·019	0·042	31
137–146 days	4	0·026	0·027	51
Lamb 1 week	2	0·080	0·046	64
2–6 weeks	3	0·106	0·054	66
Adult				
Non-pregnant	4	0·408	0·185	69
Pregnant (series 1)	8	0·175	0·079	69

Resting output of adrenaline and noradrenaline

The rate at which adrenaline and noradrenaline were released from the adrenal glands after section of the splanchnic nerves in the absence of any stimulus was low and very variable at all ages. The data summarized in Table 3 show that the proportion of adrenaline in the foetus was lower than after birth, as might be expected from the proportions of the two amines stored in the gland. After birth, there was little change in the proportions of the two amines secreted, although the absolute levels of both increased with age. During pregnancy smaller amounts of both

amines were released compared with the non-pregnant animal, but the results were obtained from the group of animals in which the pressor amine content of the glands was very low (Table 2, series 1).

Response of the adrenal medulla to splanchnic nerve stimulation

Lambs and adult sheep. The rate at which adrenaline and noradrenaline were secreted in response to splanchnic nerve stimulation was estimated in lambs from birth to 3 weeks, at 7–8 weeks of age, and in pregnant and non-pregnant adult animals. The results (Table 4) show that, as with the content of the adrenal gland, the output increased with the age and size of the glands and that there was no obvious change in the percentage of adrenaline secreted between birth and maturity. In the pregnant animals

TABLE 4. Rate of output of pressor amines from the adrenal glands of lambs and adult sheep during 2 min stimulation of the peripheral ends of the splanchnic nerves

Age	No. of animals	Mean adrenal wt. (g)	Mean rate of pressor amine output (μg/min)		Mean % adrenaline	
			Adrenaline	Noradrenaline	in effluent	in glands
Lamb						
New-born	5	0·32	0·85	0·58	60	67
1–3 weeks	4	0·36	1·92	0·98	66	
7–8 weeks	3	0·73	4·90	3·70	58	69
Adult						
Non-pregnant	6	1·9	8·38	4·20	66	71
Pregnant (series 1)						
80–140 days	18	2·1	4·04	1·29	76	86
142 days-term	4	2·5	9·91	3·94	72	83

used the response to splanchnic nerve stimulation was smaller between 80 and 140 days, and the percentage of adrenaline secreted higher, than in the non-pregnant animals, but the output of both amines returned to the non-pregnant levels at or near term. These results were, however, obtained from the glands of pregnant ewes (series 1) which had a low content of pressor amines in the adrenal gland.

Foetal lambs. The output of adrenaline and noradrenaline in response to stimulation was measured in foetal lambs from 80 days until the end of pregnancy. Before 115 days the surface of the gland was stimulated by direct application of the electrodes, thereafter they were applied to the peripheral end of the cut splanchnic nerve. The results (Fig. 2) show that the foetal adrenal medulla responded to stimulation in two distinct phases, according to the maturity of the animal. During the first phase, which lasted until about 125 days, the rate of output was very low, only 2–3 times that of the resting level and more noradrenaline than adrenaline was released. An analysis of the individual observations showed that this

difference was statistically significant ($P < 0{\cdot}001$). From about 125 days until term the rate of release of the two amines on splanchnic nerve stimulation rose sharply (Fig. 2), and after 134 days the output of adrenaline was significantly higher than that of noradrenaline ($p < 0{\cdot}01$).

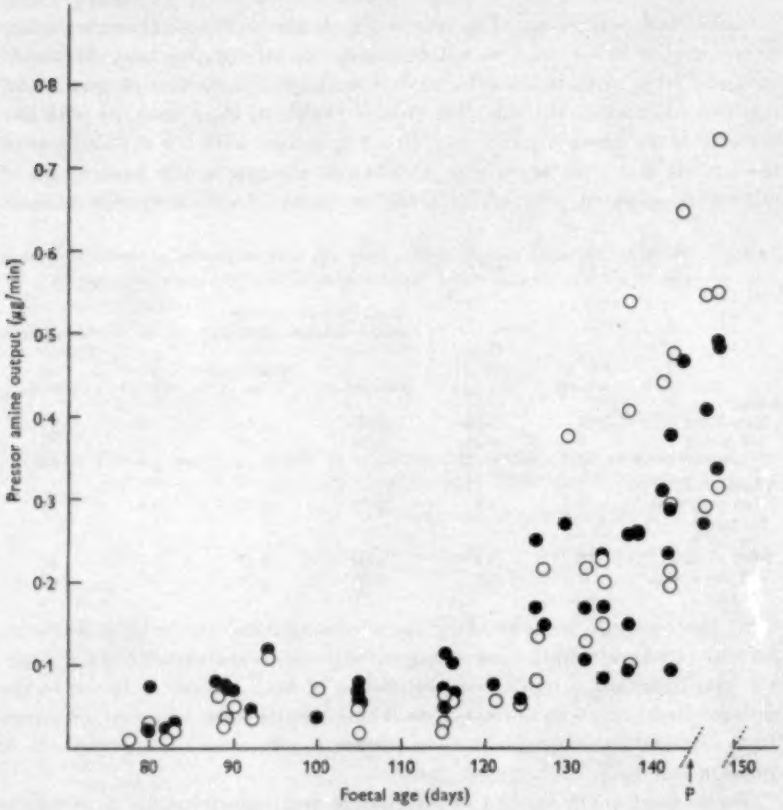

Fig. 2. The change in the rate of output of adrenaline (○), and noradrenaline (●) from the left adrenal gland of the foetal lamb with age, during stimulation, either of the surface of the gland (80–105 days) or of the peripheral ends of the cut splanchnic nerves (115 days–term). P, parturition.

These two phases could not be accounted for by the different positions of the electrodes, since direct stimulation of the surface of the glands was only used in foetuses between 80 and 105 days. Furthermore, when the efficiency of the two positions was compared in the same animal, in foetuses at 115 days of pregnancy (Fig. 3), it was found that stimulation of the splanchnic

nerves released only 0·095 μg noradrenaline and 0·03 μg adrenaline/min at this age, while 0·177 μg noradrenaline and 0·065 μg adrenaline/min were secreted when the surface of the glands was stimulated directly.

The sudden change in the rate of adrenaline and noradrenaline output in response to splanchnic nerve stimulation at about 125 days was not accompanied by any corresponding change in the weight of the adrenal glands or in their pressor amine content. It seemed probable, therefore, that the increased efficiency of this form of stimulus in late pregnancy was due to the development of the innervation of the adrenal medulla during this period: this possibility was tested with hexamethonium.

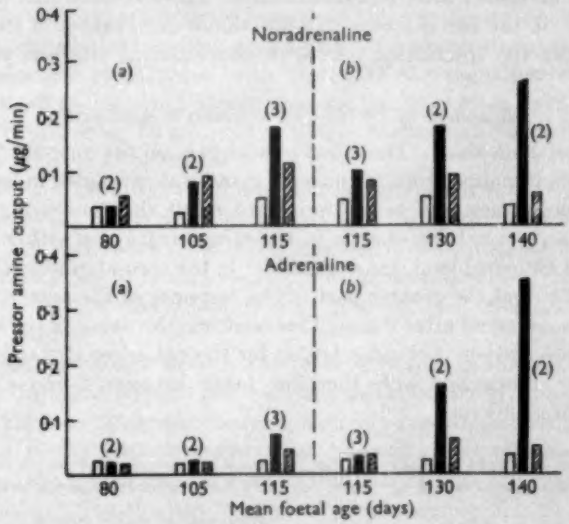

Fig. 3. The effect of hexamethonium (10 mg/kg I.V.) on the rate of pressor amine output from the left adrenal gland during (*a*) direct, surface, or (*b*) splanchnic nerve stimulation in the foetal lamb. Mean rate of output at rest □; during stimulation before (■), and after (▨) hexamethonium. Number of observations given in parentheses.

The effect of hexamethonium. This was examined only in the foetal lamb and not in animals after birth. The conditions of the experiments including the method of application of the electrodes were the same as in those described above.

Hexamethonium abolished the increasing effect of splanchnic nerve stimulation on the adrenal medulla which occurred in foetuses older than 125 days. Figure 3 shows that in these animals, the rate of output of both amines in response to stimulation was reduced to about twice the resting

level of secretion. In foetuses from 80 to 115 days of age when very small amounts of adrenaline or noradrenaline were released on stimulation, hexamethonium had little effect. However, when the output of either or both amines increased, i.e. at 115 days, injection of hexamethonium again reduced the response to about twice the resting level of secretion.

The intravenous injection of hexamethonium into the foetus usually produced a slight fall in its carotid arterial blood pressure and a decrease in heart rate, accompanied by an increase in pulse pressure; this slight effect was not seen in foetuses near term. In all animals, however, the rise in arterial blood pressure consequent upon stimulation of the splanchnic nerves was abolished after hexamethonium. These results, therefore, confirmed that, in the foetal lambs, the functional innervation of the adrenal medulla from the splanchnic nerves develops during the last 20 days of pregnancy.

Response of the adrenal medulla to asphyxia

Lambs and adult sheep. The effect of asphyxia on the output of adrenaline and noradrenaline from the adrenal gland was examined in lambs from 5 to 8 weeks of age and in non-pregnant adult sheep. During the first minute of asphyxia little change in the concentration of either amine in the adrenal effluent blood was detectable; in the second minute the results were variable and the greater part of the response of the adrenal medulla to asphyxia occurred after 2 min. This confirms the delay in the asphyxial discharge reported by Celander (1954) for the cat adrenal gland. Routine samples for comparison were therefore taken between 2 and 4 min after the onset of asphyxia.

TABLE 5. Rate of output of pressor amines during asphyxia in the 2–3 month old lamb and adult sheep; duration of sampling, 2 min, begun 1½–2 min after the onset of rebreathing N_2

	No. of animals	Splanchnic nerves	Mean rate of amine output (μg/min)		Mean % adrenaline
			Adrenaline	Noradrenaline	
Lamb	4	Intact	6·62	3·50	65
	3	Cut	0·44	0·41	52
Adult	3	Intact	12·6	5·50	70
	4	Cut	1·88	0·46	80

The rate of output of adrenaline and noradrenaline during asphyxia (Table 5) was slightly higher than during stimulation of the peripheral end of the cut splanchnic nerve, but similar proportions of the two amines were released by the two types of stimuli.

After section of the splanchnic nerves the output of both adrenaline and noradrenaline during asphyxia decreased to only about 10 % of the previous levels. These results indicate that in lambs and adult sheep asphyxia has

little direct effect upon the adrenal medulla and that the response is mediated almost entirely through the splanchnic nerves.

Foetal and newly born lambs. The output of adrenaline and noradrenaline during asphyxia was investigated in the foetal lamb from 80 days until near term, i.e. between 146 and 148 days, and also in lambs 1–5 days after birth.

The onset of asphyxia in the foetus, after tying the umbilical cord, was much more rapid than in the new-born lamb or the adult. The cardiovascular changes which occur after occlusion of the umbilical cord in the sheep foetus at different ages have been described by Dawes, Mott & Shelley (1959); similar results were found in the present series of experiments. The most characteristic feature of the circulatory changes during asphyxia, namely, the sudden appearance of a slow carotid pulse, which was not changed by atropine or by section of the vagus nerves, occurred not only in all foetuses but also in lambs a few days after birth. The pulse pressure varied from 10 mm in the young foetuses to 20–30 mm in animals near term or after birth.

The effect of asphyxia on the output of adrenaline and noradrenaline from the adrenal medulla was examined with the splanchnic nerves intact and after they had been cut. Its effect was also investigated in foetuses after the administration of hexamethonium, after destruction of the thoracic and lumbar part of the spinal cord by pithing, and after transection of the spinal cord at C1.

With intact splanchnic nerves. The rates of output of noradrenaline and adrenaline from the adrenal glands of the foetal lamb during asphyxia are shown in Fig. 4*a*. Adrenaline secretion closely resembled that induced by stimulation of the splanchnic nerves at different stages of foetal development. Thus the rate of output was low until about 125–130 days and then rose abruptly during the last 20 days of pregnancy.

Noradrenaline, however, showed quite a different pattern of secretion. The rate of output increased gradually from 80 days until term, and the sharp rise at about 125 days, found on stimulation of the splanchnic nerves, was absent, owing to the much greater response to asphyxia in foetuses from 100 days of age. These differences between the effect of asphyxia and the effect of splanchnic nerve stimulation on the discharge from the adrenal medulla are shown most clearly in Fig. 5. The results have been plotted in arbitrary groups according to age, so that mean figures for output and percentage adrenaline in several series of experiments can be compared at different stages of development.

Asphyxia (Fig. 5*c*) appeared to be a more effective stimulus for the discharge of both amines than stimulation of the cut splanchnic nerves (Fig. 5*e*), but while the rate of adrenaline secretion was only about

20% higher during asphyxia, the output of noradrenaline was 3–4 times greater than during a comparable period of splanchnic nerve stimulation. As a result the percentage of adrenaline in the effluent blood during asphyxia (Fig. 5*c*) was considerably lower in all groups than during splanchnic nerve stimulation (Fig. 5*e*) and bore little relation to the corresponding figures for the percentage of adrenaline in the adrenal glands of either asphyxiated or control animals (Figs. 1 and 5*a*).

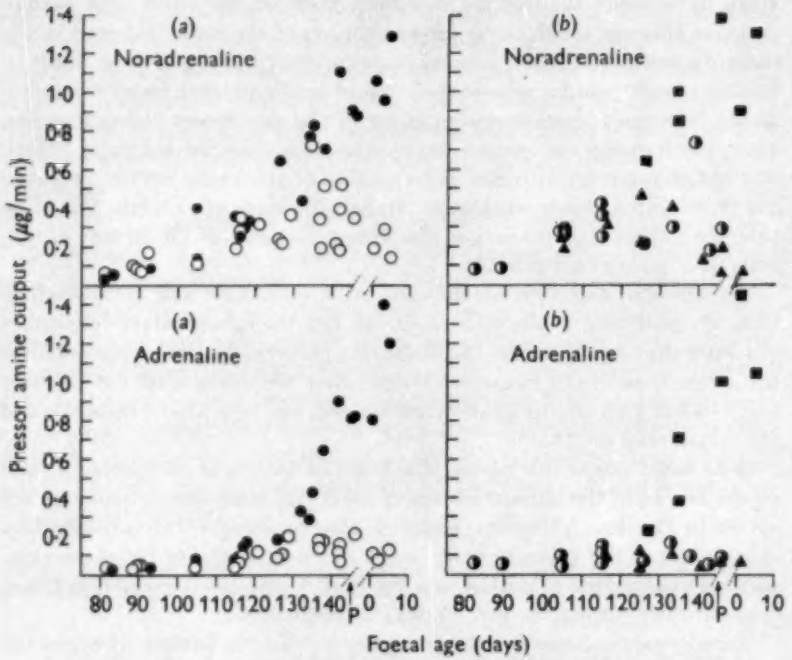

Fig. 4. The changes in the rate of adrenaline and noradrenaline output from the left adrenal gland with age, in foetal and new-born lambs during asphyxia. (*a*), response with intact innervation (●), and after cutting the splanchnic nerves (○); (*b*), response after spinal cord transection at C1 (■), after hexamethonium (◑), and after pithing spinal cord (▲). P, parturition.

After section of the splanchnic nerves. The rates of output of noradrenaline and adrenaline during asphyxia under these conditions are again shown in Fig. 4*a*. There was a considerable difference between the levels of secretion of the two amines after splanchnic nerve section.

Adrenaline output at all ages was extremely low, and in foetuses up to 115 days of age the amounts released were similar to those found during stimulation of the surface of the adrenal gland (see Fig. 5*b*, *e*). The rate

of output increased slightly towards the end of pregnancy but the maximum rarely exceeded 0·2 μg/min, which represented a decrease of at least 75 % of the amount released from the innervated adrenal gland.

Noradrenaline secretion during asphyxia after splanchnic nerve section was much higher. Until about 120 days the output was indistinguishable from that obtained with intact splanchnic nerves; thereafter there was a slight increase to a maximum of 0·4–0·5 μg/min, which represented at

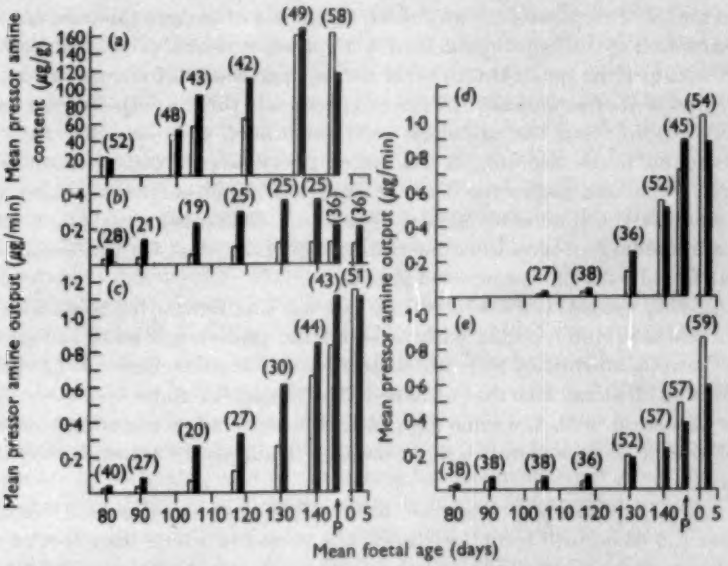

Fig. 5. Comparison between changes in pressor amine content and rate of output of the adrenal glands of foetal lambs under different conditions. □, adrenaline; ■, noradrenaline; % adrenaline in parentheses. (*a*), mean total adrenaline and noradrenaline content from glands removed after minimum interference; (*b*), mean rate of output from denervated left adrenal gland during asphyxia; (*c*) mean total rate of discharge from innervated gland during asphyxia; (*d*) response to asphyxia which may be attributed to splanchnic nerves, i.e. (*c*) minus (*b*); (*e*) mean rate of output during splanchnic nerve stimulation. P, parturition.

least 50 % of the output of the innervated gland. The response to asphyxia waned rapidly at the end of pregnancy and immediately after birth, and in the first few days of life only about 0·2 μg noradrenaline and 0·1 μg adrenaline/min were released from the denervated gland.

The effect of asphyxia on the foetal adrenal glands was, therefore, very different from that in the adult (see Tables 4 and 5). The greatest discrepancies were due to the high output of noradrenaline in the foetus,

which represented a considerable proportion of the total discharge, and which appeared to be produced as a result of the direct effect of asphyxia upon the cells of the foetal adrenal medulla. The distribution of the branches of the splanchnic nerves in the sheep is, however, very variable and it could be argued that the residual effect after section of the splanchnic nerves might have been mediated by small nerves which had not been cut. If present, such fibres would have to excite, almost specifically, noradrenaline-containing cells to give a discharge containing 70–80% of this amine. This possibility was tested by the use of hexamethonium and by destruction of the foetal spinal cord, both of which should prevent any effective action of the splanchnic or other nerves upon the foetal adrenal medulla.

Effect of hexamethonium. In some experiments this was injected immediately before tying the umbilical cord; in others, in which the effect of splanchnic nerve stimulation was tested previously, hexamethonium was given 10–20 min before the onset of asphyxia. In either case, irrespective of whether the splanchnic nerves were intact or had been cut, the release of adrenaline and noradrenaline during asphyxia (Fig. 4*b*) was similar to that found with the denervated gland.

Effect of spinal cord destruction by pithing. The results for this series of animals are given together with those for the preceding section in Fig. 4*b*. The adrenal medullary response to asphyxia after destruction of the spinal cord was identical with that obtained after hexamethonium and splanchnic nerve section, with the same high rate of noradrenaline secretion between 105 and 140 days and only a slight increase in adrenaline secretion over this period.

Effect of spinal cord transection at C1. This was carried out on foetuses from 125 days until term, i.e. during the period in which the effect of the nervous component in the response to asphyxia became evident. At these ages the procedure caused a considerable sympathetic discharge; accordingly the preparation was left for 5–10 min before ligature of the umbilical cord. The results, given in Fig. 4*b*, show that after transection of the spinal cord at this level the rate of output of both adrenaline and noradrenaline during asphyxia was indistinguishable from that in the normal foetus. Comparison of the results with those obtained after destruction of the spinal cord shows the importance of the spinal centres in this response.

DISCUSSION

Release of adrenaline and noradrenaline from the adrenal medulla

The experiments described in this paper show that the response of the foetal adrenal medulla to asphyxia is, in part, independent of its nerve supply. The most characteristic feature of this response was the very high

proportion of noradrenaline released, which amounted to about 80 %, and which bore no relation to the proportion found in the adrenal glands. The alternative explanation, that this residual component might be mediated by small nerve fibres which do not course with the main splanchnic nerves, and which had thus escaped section, is entirely unsupported by the evidence. The similarity of the response after splanchnic nerve section to that obtained after hexamethonium or after destruction of the spinal cord, and its presence early in foetal life, all point to the adrenal medulla itself as the site of the sensitive elements which are directly affected by asphyxia.

The foetal adrenal medulla, therefore, differed from that of the lamb and adult sheep, in which the response to asphyxia after splanchnic nerve section was only 1/10th of that found with the nerves intact and showed no predominant output of noradrenaline. The presence of a non-nervous response to asphyxia in the adult was first reported by Bülbring, Burn & de Elio (1948) using the perfused adrenal gland of the dog, but no further analysis of the phenomenon appears to have been made, although Vogt (1952) observed a slight increase in output of both amines from the denervated glands of a cat during asphyxia.

In the foetus a detailed study of the properties of the cells of the adrenal medulla, or of the nature of the stimulus, provided by asphyxia, which elicits the direct response, has not been undertaken. The predominance of the response before a functional nerve supply to the gland could be detected, and its subsequent decay as this developed, suggested that lack of innervation might be one of the contributory factors. The phenomenon appears to be analogous to that shown by phaeochromocytoma. Most of these tumours have a high noradrenaline content and release it either spasmodically or continuously (Holton, 1949; Goldenberg, 1951; Burger & Langemann, 1956; Cohen & Goldenberg, 1957; von Euler, 1958). They are assumed to lack innervation and whether asphyxia of the cells due to growth of the neoplasm is responsible for the high output, occasionally paroxysmal in nature, remains to be shown.

Clear evidence for the development of the functional innervation of the foetal adrenal medulla during the last 20 days of pregnancy was provided by the results with splanchnic nerve stimulation. An abrupt rise in the rate of output of both amines occurred from about 125 days onward with this form of stimulation and the response was almost completely annulled by hexamethonium. The proportions of the two amines released on stimulation were similar to those found in the glands removed after minimum stress.

The abrupt development of innervation at 125–130 days was also reflected in the change in the amount and composition of the total asphyxial discharge at this stage. Thereafter the divergence between the two components of the response to asphyxia increased, and the effects of the

splanchnic nerves predominated at the end of pregnancy. Nevertheless, at no time during foetal life was the total discharge during asphyxia similar to that induced by splanchnic nerve stimulation, either in composition or amount (see Fig. 5*c*, *e*), and it was the presence of the residual component, which was independent of the nerve supply, which largely accounted for this anomaly. When this was subtracted from the total asphyxial discharge the remainder of the response, mediated by the splanchnic nerves (Fig. 5*d*), was then somewhat similar to that obtained during stimulation of the peripheral ends of the cut nerves (Fig. 5*e*). However, even after the elimination of the direct effect upon the gland, a disproportionately large amount of noradrenaline was still released by asphyxia.

In the lamb and adult sheep, on the other hand, there was no such consistent difference between the discharge induced by asphyxia from the innervated gland and that elicited by direct splanchnic nerve stimulation. In both cases the percentage of adrenaline released was similar to that found in the glands. These results on the adult sheep are thus in agreement with the findings in other adult animals (Lund, 1951; von Euler & Folkow, 1953; Rapela, 1956).

The spinal cord alone, and not the remainder of the brain stem, appeared to be essential for the nervous response to asphyxia in the foetal and new-born lamb, since the results obtained after transection at C1 were indistinguishable from those in the intact animal. In the adult animal sympathetic centres in the medulla oblongata and hypothalamus are generally assumed to be activated in any discharge of the adrenal medulla, including its response to asphyxia (Elliott, 1912; Houssay & Mollinelli, 1926; Redgate & Gelhorn, 1953; von Euler, 1956). These areas of the brain stem are certainly more sensitive to asphyxia than the spinal vasomotor centres (Mathison, 1910, 1911), but Jourdan (1936) has provided evidence for the participation of the cervical region of the spinal cord in the response of the adrenal glands to asphyxia, and Tenney (1956) has also found that with high CO_2 concentrations the discharge measured by the contraction of the denervated nictitating membrane was almost as great in the spinal cat as in the intact anaesthetized animal. Whether the spinal cord of the foetal and new-born lamb is more sensitive to asphyxia or less liable to spinal shock than that of the adult is not known. But it may be that the major part of the adrenal medullary discharge during asphyxia is always dependent on the spinal centres, while the brain stem primarily mediates the more delicately adjusted vasomotor reflexes of the sympathetic system.

The development of innervation in the foetal adrenal gland of the sheep during the last three weeks of pregnancy agrees with the results and conclusions of Born *et al.* (1956) on the increased activity of the autonomic system as a whole during asphyxia of the foetal lamb near term. The

maturity of the adrenal medulla at birth is further indicated by the fact that the proportions of adrenaline and noradrenaline in both glands and effluent blood in young lambs were similar to those of the adult, and only the absolute levels increased with age. Thus, in this respect, the young lamb may be considered as a miniature adult. Other evidence suggests that this early development of the sympathetic system probably does not apply to other species. Bauer (1938) was able to detect sympathetic nervous activity in the rabbit during asphyxia only after birth. The lamb also contrasts sharply with the calf where the effects of splanchnic nerve stimulation in the new-born animal are small (Comline & Titchen, 1951) and the major developmental changes in the output and content of the adrenal medulla occur after birth (Silver, 1960). In other species, therefore, the direct response of the cells of the adrenal gland to asphyxia may be of considerable importance at birth, and even in the sheep foetus it may play a part in the survival of premature animals, since viable lambs have been obtained as early as 110 days (Born, Dawes & Mott, 1955) when the nervous mechanism is not yet established.

Dawes *et al.* (1959) have concluded that maintenance of the circulation is of prime importance for survival of foetal and new-born animals after prolonged asphyxia, and the pressor amines released from the adrenal medulla under these conditions may be essential for this purpose. On the other hand bilateral adrenalectomy is not followed by circulatory failure during asphyxia nor does it prevent the development and maintenance of the slow cardiac rhythm which is so characteristic of foetal asphyxia (Comline & Silver, unpublished observations). It is not possible, therefore, to define the precise role of the large amount of noradrenaline discharged from the foetal adrenal medulla during asphyxia. It might stimulate heat production in foetal and new-born lambs in a manner similar to that described for the kitten (Moore & Underwood, 1960*a*, *b*), and it may be significant that noradrenaline is a more effective calorigenic agent than adrenaline in these animals and that its potency declines with age.

Adrenaline and noradrenaline content of the adrenal glands

In this examination of the development of the adrenal glands of the sheep a marked decrease in the content of both amines was found in glands obtained from animals which had been subjected to death from asphyxia or to considerable operative trauma, as compared with those removed with minimum interference after a short period of anaesthesia. This discrepancy, affecting noradrenaline more than adrenaline, was at first ascribed to endogenous discharge, and it appeared to provide evidence for the existence of a labile store of noradrenaline which might be readily released by asphyxia or by other stimuli. Closer examination of the

differences between the two groups showed that the apparent losses were far greater than could be accounted for solely by the known maximum rates of secretion either in the foetus or in the ewe. The discrepancies between the two sets of observations were not related to differences in adrenal weight nor to development of the adrenal cortex, and it seemed unlikely that they were due to a consistent error in the extraction procedure or assay, since no discrepancies in other estimations were detected. Butterworth & Mann (1957, 1960) have reported considerable variation in the pressor amine content of cat adrenal glands and only found close agreement in related animals. This may well apply to the sheep, since the material for the two series of ewes and foetuses was not obtained during the same year nor from an inbred population. Furthermore, the unaccountable variations in content between different animals, which were found even when precautions were taken to minimize endogenous discharge, might also be related to the condition of the animals or the particular strain used. The apparent inherent variability of the pressor amine content of adrenal glands is also illustrated by the table compiled by von Euler (1956) which summarizes figures for 'normal' glands obtained in different laboratories. The values given for pressor amine concentration in the sheep vary from 0·23 to 0·91 mg. noradrenaline and 0·75–1·50 mg adrenaline/g adrenal tissue, the percentage of adrenaline ranging from 56 to 81%.

In view of this variability in adrenal gland content, the value of such estimations as indices of the degree of development or activity of the glands would seem to be very dubious without many qualifications or unless they are combined with direct measurements of discharge from the glands.

SUMMARY

1. The output of adrenaline and noradrenaline in response to asphyxia or splanchnic nerve stimulation has been examined in foetal lambs from 80 days until term, in lambs and in adult sheep.

2. Stimulation of the splanchnic nerves in the foetus had little effect until about 125 days; thereafter the response increased abruptly until term and released a percentage of adrenaline similar to that found in the glands from non-asphyxiated animals. This response was annulled by hexamethonium.

3. The response to asphyxia of the foetal adrenal medulla consisted of two components: these were (i) a direct effect upon the adrenal medulla and (ii) that mediated through the splanchnic nerves. The latter was only present during the last 15–20 days of pregnancy.

4. The direct response to asphyxia typically released 80% of noradrenaline; it could be detected before the innervation of the gland was established and was obtained after injecting hexamethonium or pithing of the

spinal cord. It was present throughout the period of pregnancy examined and only waned a few days before birth.

5. The total discharge of the innervated gland during asphyxia in the foetus was considerably higher and the percentage of noradrenaline greater than that elicited by splanchnic nerve stimulation or contained in the gland. This discrepancy was largely accounted for by the direct effect of asphyxia upon the foetal adrenal medulla.

6. After transection of the spinal cord at C 1 the response to asphyxia was indistinguishable from that in the normal intact foetus and new-born lamb.

7. In the lamb a few days after birth the percentage of adrenaline in the glands and in the effluent blood during stimulation of the splanchnic nerves was similar to that in the adult. The glandular content and the rate of secretion increased with age.

8. In the adult, in contrast to the foetus, the direct effect of asphyxia on the adrenal medulla amounted to only 10 % of the discharge from the innervated gland and there was no predominant output of noradrenaline. The percentage of adrenaline released by both innervated and denervated glands during asphyxia was similar to that in the gland.

9. The effect of different experimental procedures on the adrenaline and noradrenaline content of adrenal glands from foetuses, lambs and adult sheep was examined. The extensive depletion of the glands which occurred after death from asphyxia, or after severe operative trauma, could only in part be ascribed to endogenous discharge.

We wish to thank Mr W. S. Mansfield for help with the purchase of the ewes, Messrs G. E. Baker and P. Hughes for technical assistance and Mr J. Smith for his care of the animals.

REFERENCES

BAUER, D. J. (1938). The effect of asphyxia upon the heart rate of rabbits at different ages. *J. Physiol.* **93**, 90–103.

BORN, G. V. R., DAWES, G. S. & MOTT, J. C. (1955). The viability of premature lambs. *J. Physiol.* **130**, 191–212.

BORN, G. V. R., DAWES, G. S. & MOTT, J. C. (1956). Oxygen lack and autonomic nervous control of the foetal circulation in the lamb. *J. Physiol.* **134**, 149–166.

BÜLBRING, E., BURN, J. H. & DE ELIO, F. J. (1948). The secretion of adrenaline from the perfused suprarenal gland. *J. Physiol.* **107**, 222–232.

BURGER, M. & LANGEMANN, H. (1956). Bestimmungen von Adrenalin und Noradrenalin sowie von Decarboxylase-und Aminoxydase-Aktivitäten in Zellfraktionen von Phäochromocytomen. *Klin. Wschr.* **34**, 941–944.

BUTTERWORTH, K. R. & MANN, M. (1957). A quantitative comparison of the sympathomimetic amine content of the left and right adrenal glands of the cat. *J. Physiol.* **136**, 294–299.

BUTTERWORTH, K. R. & MANN, M. (1960). Proportion of noradrenaline to adrenaline in the adrenal glands of litter-mate cats. *Nature, Lond.*, **187**, 785.

CELANDER, O. (1954). The range of control exercised by the sympathico-adrenal system. *Acta physiol. scand.* **32**, Suppl. 116.

COHEN, G. & GOLDENBERG, M. (1957). The simultaneous fluorimetric determination of adrenaline and noradrenaline in plasma—II. Peripheral venous plasma concentrations in normal subjects and in patients with pheochromocytoma. *J. Neurochem.* **2**, 71–80.

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REFERENCES

BAUER, D. J. (1938). The effect of asphyxia upon the heart rate of rabbits at different ages. *J. Physiol.* **93**, 90–103.

BORN, G. V. R., DAWES, G. S. & MOTT, J. C. (1955). The viability of premature lambs. *J. Physiol.* **130**, 191–212.

BORN, G. V. R., DAWES, G. S. & MOTT, J. C. (1956). Oxygen lack and autonomic nervous control of the foetal circulation in the lamb. *J. Physiol.* **134**, 149–166.

BÜLBRING, E., BURN, J. H. & DE ELIO, F. J. (1948). The secretion of adrenaline from the perfused suprarenal gland. *J. Physiol.* **107**, 222–232.

BURGER, M. & LANGEMANN, H. (1956). Bestimmungen von Adrenalin und Noradrenalin sowie von Decarboxylase-und Aminoxydase-Aktivitäten in Zellfraktionen von Phäochromocytomen. *Klin. Wschr.* **34**, 941–944.

BUTTERWORTH, K. R. & MANN, M. (1957). A quantitative comparison of the sympathomimetic amine content of the left and right adrenal glands of the cat. *J. Physiol.* **136**, 294–299.

BUTTERWORTH, K. R. & MANN, M. (1960). Proportion of noradrenaline to adrenaline in the adrenal glands of litter-mate cats. *Nature, Lond.*, **187**, 785.

CELANDER, O. (1954). The range of control exercised by the sympathico-adrenal system. *Acta physiol. scand.* **32**, Suppl. 116.

COHEN, G. & GOLDENBERG, M. (1957). The simultaneous fluorimetric determination of adrenaline and noradrenaline in plasma—II. Peripheral venous plasma concentrations in normal subjects and in patients with pheochromocytoma. *J. Neurochem.* **2**, 71–80.

COMLINE, R. S. & SILVER, M. (1958). Response of the adrenal medulla of the sheep foetus to asphyxia. *Nature, Lond.*, **181**, 283–284.

COMLINE, R. S. & TITCHEN, D. A. (1951). Reflex contraction of the oesophageal groove in young ruminants. *J. Physiol.* **115**, 210–226.

CRAWFORD, T. B. B. & OUTSCHOORN, A. S. (1951). The quantitative separation of adrenaline and noradrenaline in biological fluids and tissue extracts. *Brit. J. Pharmacol.* **6**, 8–19.

DAWES, G. S., HANDLER, J. J. & MOTT, J. C. (1957). Some cardiovascular responses in foetal, new-born and adult rabbits. *J. Physiol.* **139**, 123–136.

DAWES, G. S., MOTT, J. C. & SHELLEY, H. J. (1959). The importance of cardiac glycogen for the maintenance of life in foetal lambs and new-born animals during anoxia. *J. Physiol.* **146**, 516–538.

ELLIOTT, T. R. (1912). The control of the suprarenal glands by the splanchnic nerves. *J. Physiol.* **44**, 374–409.

ELMES, P. C. & JEFFERSON, A. A. (1942). The effect of anaesthesia on the adrenaline content of the suprarenal glands. *J. Physiol.* **101**, 355–361.

GOLDENBERG, M. (1951). Adrenal medullary function. *Amer. J. Med.* **10**, 627–641.

HÖKFELT, B. (1952). Noradrenaline and adrenaline in mammalian tissues. *Acta physiol. scand.* **25**, Suppl. 92.

HOLTON, P. (1949). Noradrenaline in adrenal medullary tumours. *Nature, Lond.*, **163**, 217.

HOLTON, P. (1951). High concentration of noradrenaline in calves' supra-renals. *Nature, Lond.*, **167**, 858–859.

HOUSSAY, B. A. & MOLLINELLI, E. A. (1926). Adrenal secretion produced by asphyxia. *Amer. J. Physiol.* **76**, 538–550.

JOURDAN, F. (1936). Contribution à la topographie du système nerveux adrénalino- sécréteur. Présence de centres adrénalino-sécréteurs dans la moelle cervicale haute chez le chien. *Arch. int. Pharmacodyn.* **53**, 71–79.

LUND, A. (1951). Release of adrenaline and noradrenaline from the suprarenal gland. *Acta pharm. tox. Kbh.* **7**, 308–320.

MATHISON, G. C. (1910). The action of asphyxia on the spinal animal. *J. Physiol.* **41**, 416–449.

MATHISON, G. C. (1911). The effects of asphyxia upon medullary centres. Part 1. The vasomotor centre. *J. Physiol.* **42**, 283–300.

MOORE, R. E. & UNDERWOOD, M. C. (1960*a*). Noradrenaline as a possible regulator of heat production in the new-born kitten. *J. Physiol.* **150**, 13–14*P*.

MOORE, R. E. & UNDERWOOD, M. C. (1960*b*). The calorigenic action of noradrenaline in the new-born kitten: its inhibition by hypoxia. *J. Physiol.* **152**, 52–53*P*.

OUTSCHOORN, A. S. (1952). The hormones of the adrenal medulla and their release. *Brit. J. Pharmacol.* **7**, 605–615.

RAPELA, C. E. (1956). Differential secretion of adrenaline and noradrenaline. *Acta physiol. latinoamer.* **6**, 1–14.

REDGATE, E. S. & GELHORN, E. (1953). Nature of sympathetico-adrenal discharge under conditions of excitation of central autonomic structures. *Amer. J. Physiol.* **174**, 475–480.

SHEPHERD, D. M. & WEST, G. B. (1951). Noradrenaline and the suprarenal medulla. *Brit. J. Pharmacol.* **6**, 665–674.

SILVER, M. (1954). The output of adrenaline and noradrenaline from the adrenal glands of the calf. *J. Physiol.* **125**, 45–46*P*.

SILVER, M. (1960). The output of adrenaline and noradrenaline from the adrenal medulla of the calf. *J. Physiol.* **152**, 14–29.

TENNEY, S. M. (1956). Sympathico-adrenal stimulation by carbon-dioxide and the inhibitory effect of carbonic acid on epinephrine response. *Amer. J. Physiol.* **187**, 341–346.

VOOT, M. (1952). The secretion of the denervated adrenal medulla of the cat. *Brit. J. Pharmacol.* **7**, 325–330.

VON EULER, U. S. (1956). *Noradrenaline*. Springfield, Illinois: Thomas.

VON EULER, U. S. (1958). Adrenal medullary and other chromaffine cell tumours. In *Colloquia on Endocrinology*, Vol. 12, pp. 268–277. London: J. and A. Churchill.

VON EULER, U. S. & FOLKOW, B. (1953). Einfluss verschiedener afferenter Nervenreize auf die Zusammensetzung des Nebennierenmarkinkretes bei der Katze. *Arch. exp. Path. Pharmak.* **219**, 242–247.

VON EULER, U. S. & FLODING, I. (1955). A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta physiol. scand.* **33**, Suppl. 118, 45–56.

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THE STATIC ELASTIC PROPERTIES OF THE ARTERIAL WALL

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The mechanical properties of the arteries have been extensively studied in the past, but precise quantitative knowledge of this subject is still lacking. In addition, the available data are derived almost entirely from experiments on the thoracic aorta and it is not certain how the smaller vessels behave.

Much previous work describes the relation between pressure and volume in intact vessels. Pressure, radius and wall-tension in an elastic tube are all interdependent and in most cases insufficient data are given to calculate the exact behaviour of the wall itself. In addition, the retraction seen when an artery is removed from the body (Fuchs, 1900) has been neglected. It is consequently often difficult to infer the behaviour of the vessels *in vivo*, the more so as in some cases the inflated vessel was free to lengthen, while in others the specimens were held at some unspecified length. Studies have also been made on strips and rings cut from arteries but the properties of these specimens give but little information on the relation between pressure and radius in the intact vessel.

For these reasons it was felt that the question merited reinvestigation and an apparatus was developed to determine the pressure–radius relationships of arterial specimens. This was designed to enable both static and dynamic behaviour to be measured.

THE ELASTIC MODULUS

Classical elastic theory was developed to describe the relations between applied forces and the resulting deformations in relatively stiff materials such as metals. A perfectly elastic or Hookean substance shows a constant proportionality (within certain limits) between stress (applied force per unit area) and strain (relative change in dimension). This proportionality is expressed in the familiar elastic moduli such as Young's modulus (E), the ratio of tensile stress to tensile strain. Soft animal tissues are very much more extensible than metals and do not show a constant ratio between

stress and strain. To deal with these materials an entirely different theoretical approach has been advocated and these methods have been applied to the study of tissue elasticity with some success (King & Lawton, 1950). Nevertheless, this method has not been used here, largely because it rests on the assumption that such tissues are homogeneous in structure, which is patently not the case for blood vessels. The computations involved are also very lengthy.

The modulus used here is termed the incremental modulus (following a suggestion of Krafka, 1939), and strain is defined as the ratio of change in length to the mean length during the change.

The Young's modulus of an isotropic tube, which does not change in length on inflation, is given by Love (1927)

$$E = \frac{\Delta p}{\Delta R_0} \times \frac{2(1-\sigma^2)R_i^2 R_0}{(R_0^2 - R_i^2)},$$

where ΔR_0 is the change in external radius following a pressure change Δp, R_i is the internal radius and σ is known as Poisson's ratio. This is the ratio of transverse to longitudinal strain, all materials becoming narrower when they are stretched in length. If $\sigma = 0{\cdot}5$ no change in the volume of the material occurs for a very small strain. This is not strictly true when large strains are considered (Bergel, 1960), but σ has been taken as 0·5 for the arterial wall which has been shown (Lawton, 1954) to extend isovolumetrically. This equation assumes isotropy of the material, that is that the mechanical properties are identical in all directions. There is good evidence (Fenn, 1957) that the arterial wall is more extensible longitudinally than circumferentially, but when no change in length occurs the effective circumferential modulus (measured in these experiments) is a function only of the true radial and circumferential moduli (Lambossy & Müller, 1954).

With these assumptions the incremental modulus is

$$E_{\text{inc } p_2} = \frac{p_3 - p_1}{R_{0_3} - R_{0_1}} \cdot \frac{2(1-\sigma^2)R_{i_2}^2 R_{0_2}}{(R_0^2 - R_i^2)},$$

where the subscripts 1, 2, 3 represent successive measurements of pressure and radius. If no volume change occurs in the wall, then $R_0^2 - R_i^2$ is constant. The units of stress are force per unit area. Strain is a ratio of length and is dimensionless, thus the elastic modulus has the same units as stress, here given in dynes/cm^2 for 100% elongation. It is therefore necessary to measure the internal pressure, radius and wall thickness of the arteries used.

METHODS

The method chosen to measure radius changes was a photo-electric one: this has been described elsewhere (Bergel, 1958, 1961). The vessels used (thoracic, abdominal aorta, femoral and carotid arteries) were taken from the bodies of dogs which had been bled as donors for perfusion experiments; all specimens were removed within 2 hr of death and the experiments were carried out on the same day. Before and after excision the length of the specimen was measured. Chosen lengths of vessel, about 6 cm excised length, were gently cleaned of all surrounding tissue. The limit of the adventitia was arbitrarily taken as the zone reached in this way when the specimen appeared to be clean and without obvious fringes of connective tissue. Portions were selected with as few branches as possible, but all branches included were ligated and cut close to the parent vessel. Finally the artery was painted with Indian ink to reduce surface reflexions.

The cleaned specimen was weighed twice, in air and in water; thus its volume was obtained (immersion in water lasted a few seconds only and resulted in no detectable increase in weight). It was then attached at either end to a pair of tubular metal holders of suitable size, which could be screwed into two rigid horizontal supports. The specimen was then held vertically and could be stretched to its *in vivo* or natural length. In this position it lay in the path of a collimated light beam $\frac{1}{4}$ in. (3 mm) deep and of adjustable width. An accumulator supplied a 12 V 60 W filament bulb, which was separated from the specimen by two collimating slits 12 in. (25 cm) apart.

The shadow of the artery was thrown on a small diffusing screen formed by the flat surface of a length of cylindrical Perspex rod ($1\frac{1}{2}$ in. (3 cm) diam.) which had been longitudinally bisected. The flat face had been ground with fine emery powder; the curved surface acted as a lens and concentrated the light on the photo-cathode of a photomultiplier tube (R.C.A. 931 A) mounted 2 in. (5 cm) away in a light-tight box.

The photomultiplier was supplied from a stabilized E.H.T. source at a potential of 600 V (by under-running the tube somewhat the stability is improved). The last of the chain of electrodes, the anode, was connected to earth through a total load of 2·5 MΩ, and the potential of this point was taken to a high-gain oscilloscope. The maximum current drawn from the photomultiplier was 4 μA. The greater the radius of the specimen the less the amount of light falling on the photomultiplier window and the smaller the negative potential appearing on the anode. For the purpose of calibration this potential could be balanced against that from a ten-turn potentiometer which was graduated to 1000 units. Before and after each experiment the unit was calibrated with a series of metal rods of known radius mounted in the position of the artery; a linear relation between radius and output was obtained.

Procedure. While preparing the specimen the apparatus was switched on and left for 2 hr before making any measurements. Further to reduce drift, the battery supplying the lamp was under constant charge and the leads to the light were made of aluminium strips to lessen thermal resistance changes. It was not possible to eliminate drift and the apparatus was calibrated before and after each experiment. The over-all error in the measurement of radius was ±4 % over 2 hr; the great majority of experiments lasted about 1 hr and none took more than $1\frac{1}{2}$ hr.

Having mounted the specimen the static pressure–radius relationships were measured. By 'static' is meant that the radius at each step of 20 mm Hg between 0 and 240 mm Hg was measured, with a pause of 2 min after each change before balancing the output against the graduated potentiometer. Usually no further change in size was perceptible after this interval, but occasionally a longer pause was necessary. These pressure changes were produced with a hand bulb and reservoir bottle connected to the specimen through the lower support and were measured with a mercury manometer. The apparatus was filled throughout with isotonic saline. All experiments were carried out at room temperature (18–22° C).

RESULTS

The mean values for the incremental moduli of four types of artery up to a pressure of 240 mm Hg are shown in Fig. 1. The vertical lines represent the standard errors of the means at pressures of 100 and 220 mm Hg for the thoracic aorta and the carotid artery, the errors for the other vessels were similar to those of the carotid. In Table 1 these mean values and

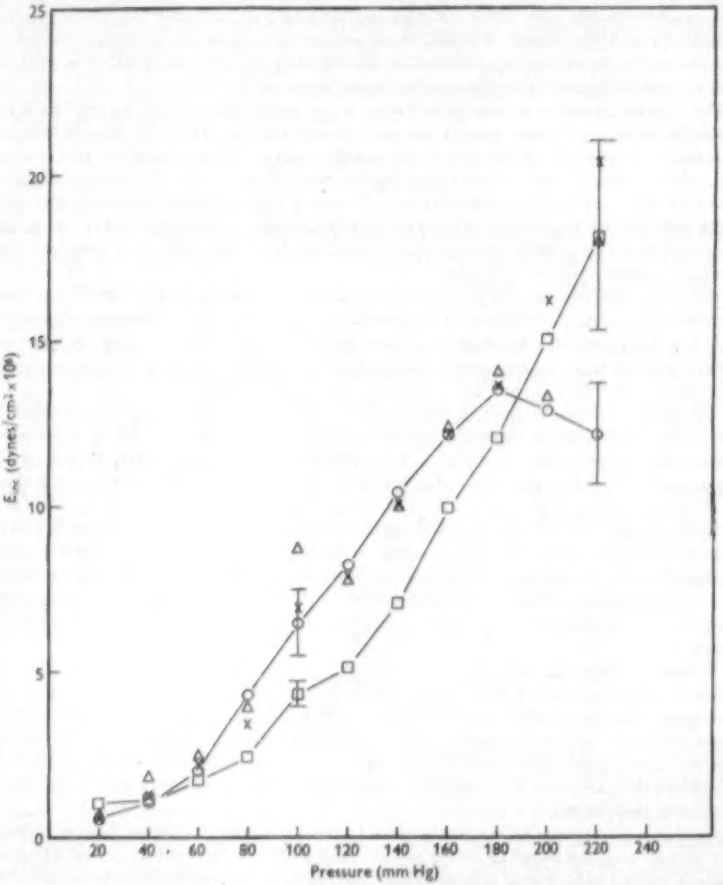

Fig. 1. Mean values for the static incremental modulus (E_{inc}) of four types of artery. Lines have been drawn connecting the points for the thoracic aorta and carotid artery; the standard errors of the means for these vessels are shown for pressures of 100 and 220 mm Hg. □ thoracic aorta; △ abdominal aorta; × femoral artery; ○ carotid artery.

standard errors are listed; the differences between the thoracic aorta and carotid artery at 100 and 220 mm Hg are probably significant ($P < 0.05$).

In all cases the vessel wall becomes progressively less extensible (higher elastic modulus) the more it is stretched. The increase in modulus is steady throughout the whole range: there is no suggestion that at some well defined pressure some less extensible material in the wall which had previously been slack is brought under strain. Up to pressures around 100 mm Hg this change is less marked in the thoracic aorta, but thereafter the modulus rises rapidly and becomes essentially the same as that of the other vessels. In the case of the carotid artery, but not of the other vessels,

TABLE 1. Mean values for static incremental modulus of elasticity (dynes/cm^2 $\times 10^6 \pm$ S.E. of mean)

Pressure (mm Hg)	Thoracic aorta	Abdominal aorta	Femoral artery	Carotid artery
40	1·2 ± 0·1 (6)	1·6 ± 0·4 (4)	1·2 ± 0·2 (6)	1·0 ± 0·2 (7)
100	4·3 ± 0·4 (12)	8·9 ± 3·5 (8)	6·9 ± 1·0 (9)	6·4 ± 1·0 (12)
160	9·9 ± 0·5 (6)	12·4 ± 2·2 (4)	12·1 ± 2·4 (6)	12·2 ± 2·7 (7)
220	18·1 ± 2·8 (5)	18·0 ± 5·5 (3)	20·4 ± 4·4 (6)	12·2 ± 1·5 (7)

The number of measurements is shown in brackets. Some additional specimens were studied at 100 mm Hg before making dynamic measurements, and these have been included.

a limiting modulus of the order of 13×10^6 dynes/cm^2 appears to be reached at extreme distension. (These figures are the differentials of a very steeply rising stress–strain curve and the small decrease may not be a real one.)

In all these experiments the length of the vessel was held constant; it is thus simple to construct a pressure–volume curve. A roughly linear relation between pressure and volume in the thoracic aorta was found up to pressures of 100–120 mm Hg. The pressure, wall thickness, and vessel radius determine the wall tension, but the pressure–volume curve will be a straight line if the wall modulus increases proportionally with the fourth power of the radius (Bergel, 1960). This is the situation in the thoracic aorta below 100 mm Hg, but for the other vessels the increase in modulus is steeper. At high pressures the volume distensibility ($\Delta V/\Delta p$) of the thoracic aorta becomes progressively less. The other vessels show steadily diminishing distensibility throughout the whole pressure range. The behaviour of these thoracic specimens contrasts with that reported by Roy (1880), Wagner & Kapal (1951) and, for young specimens, by Hallock & Benson (1937), all of whom found markedly sigmoid pressure–volume curves. The first two of these reports refer to experiments in which the vessel was allowed to lengthen on inflation, while Hallock & Benson appear to have held their vessels at excised length during a rapid (*ca.* 2 mm Hg/sec) inflation. In another experiment these procedures were

carried out on the same specimen of thoracic aorta. The vessel was inflated slowly (2 min between each increment of 20 mm Hg) while held at its natural length, and again while free to lengthen, and finally a rapid inflation (0–300 mm Hg in 3 min) was performed at the excised length. The experiments were performed in the order given and 2 hr separated them to allow the vessel to recover fully from the stretch.

The results (Fig. 2) show that the double curvature of the pressure–volume curve is much accentuated by allowing it to lengthen, or by rapid inflation. Roy (1880) and Wagner & Kapal (1951) have emphasized that

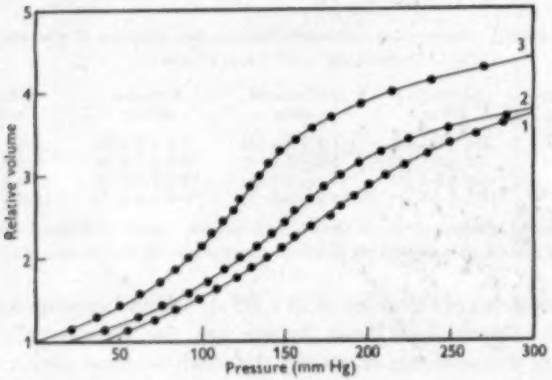

Fig. 2. Three pressure–volume curves for the same thoracic aorta. (For clarity, curves 1 and 2 are displaced horizontally.) 1, slow inflation, held at natural length; 2, fast inflation, held at natural length; 3, slow inflation, free to lengthen. Note the markedly sigmoid form of curve No. 3 which results from the vessel being free to lengthen as it is inflated. This curve also shows the greatest over-all distensibility.

the aorta is most distensible in the physiological pressure range. This effect, which is largely due to artifacts, can be seen in the high slope of the distensibility curve (No. 3) in this region.

During successive inflations all vessels became progressively larger. This tendency was particularly marked in the muscular vessels and it was normally reduced as far as possible by inflating the vessel to 250 mm Hg for a few minutes before each experiment, allowing the specimen to stretch and thus reducing the subsequent slow dilatation.

By omitting this preliminary stretch it was possible to make measurements on the same vessel over a wide range of diameter. The results of one such experiment are shown in Figs. 3 and 4. Figure 3 shows three pressure–volume curves obtained from the same specimen of femoral artery. These have been calculated from the measurements of external

radius. The first cycle of inflation and deflation was performed on a vessel which had not been under pressure since the death of the animal some hours previously. This cycle resulted in a wide loop, and at the end the zero-pressure volume had increased by some 50%. After being held at a steady internal pressure of 150 mm Hg for 20 min, during which time a further increase in size occurred, a second cycle was carried out. Again the vessel became larger. After dynamic measurements (mean pressure of 100 mm Hg for 20 min) a final slow inflation and deflation followed.

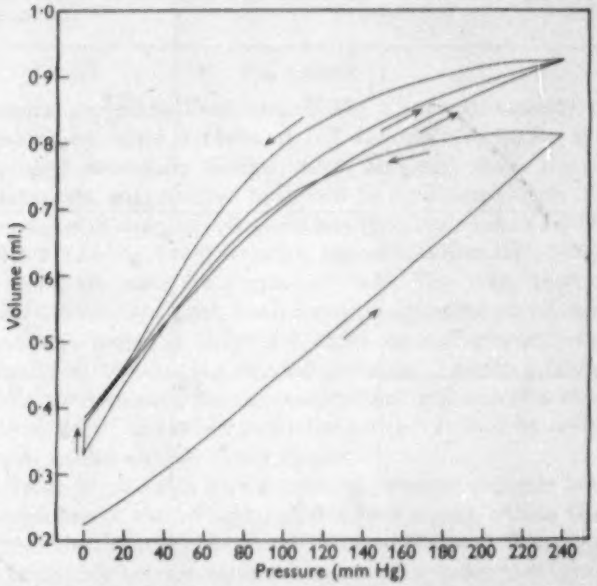

Fig. 3. Three consecutive pressure–volume curves of a femoral artery. The sequence of inflation and deflation is shown by the arrows (the last two deflations followed the same course). The vessel appears to become rather less distensible during the series of inflations (cf. Fig. 4).

This time the loop was closed (the course of deflation was identical in the second and third cycles) and no further increase in size occurred. This gradual increase in size accompanied by narrowing of the hysteresis loop has been observed by many workers (e.g. Remington, 1955).

The same data, for the inflation limbs only, are plotted as elastic modulus and radius in Fig. 4. All the points lie about a single curve, though the wall was somewhat stiffer on the first occasion. However, the modulus at any given pressure increased with each inflation. Points referring to 100 mm Hg are indicated by vertical lines and the modulus

increased from 1·1 to 4·8 × 10^6 dynes/cm². In the first cycle the vessel was less stiff because it was smaller. This experiment was repeated on other vessels with similar results.

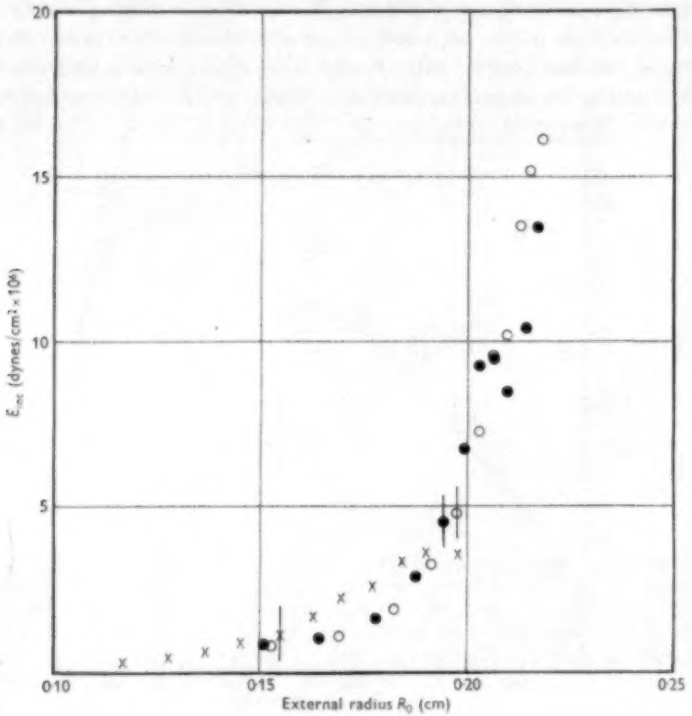

Fig. 4. The variation with radius of incremental modulus calculated from the inflation limbs of the three curves shown in Fig. 3. The vertical lines indicate points at 100 mm Hg pressure. × first inflation; ● second inflation; ○ third inflation. Note the consistency in behaviour seen when the results of the experiment illustrated in Fig. 3 are plotted in this fashion.

Measurements were also made of the amount of shortening on excision, and of the relative wall thickness (h/R_0) at an internal pressure of 100 mm Hg. Mean values are shown in Table 2. The wall-thickness figures were computed from measurements of external radius and the wall volume of the specimens. A few subsequent measurements on cut sections of vessels fixed while distended gave the same results.

TABLE 2. Mean values for relative wall thickness (h/R_0) and amount of retraction in different dogs' arteries

Vessel	h (% of R_0 at 100 mm Hg internal pressure)	Retraction (shortening as % original length)
Thoracic aorta	10·5 ± 0·46 (15)	31·9 ± 0·6 (22)
Abdominal aorta	10·5 ± 0·61 (9)	34·0 ± 0·6 (17)
Femoral artery	11·5 ± 0·96 (11)	42·0 ± 0·9 (22)
Carotid artery	13·2 ± 0·75 (13)	35·0 ± 0·5 (29)
Iliac artery	—	39·5 ± 0·7 (9)

The variations shown are the S.E. of means. Figures in brackets are the number of measurements.

DISCUSSION

The arterial properties illustrated in Fig. 1 are qualitatively similar to those reported by other workers. A full discussion of earlier studies has been published previously (Bergel, 1960) and only those reports which provide adequate quantitative data will be considered here. The longitudinal modulus of the thoracic aorta has been reported as 1·5 (Hardung, 1953) and 2–3 (Lawton, 1955) for strips, and as 2 (McDonald, 1960) for whole vessels; where all units are dynes/cm^2 × 10^6. The value reported here, 4·3 ± 0·4 is 1·5–2 times as great, but it has been estimated previously (Bergel, 1960) that this vessel is about 1·5 times as stiff circumferentially as longitudinally at 100 mm Hg internal pressure. Lawton's (1960) figures of about 2 × 10^6 dynes/cm^2 for the longitudinal modulus of a wide variety of arteries refer to somewhat retracted strips. It can be seen that all vessels have similar moduli at low strains.

The increase in modulus with increasing pressure depends both on the elastic properties of the collagen, elastin and muscle within the arterial wall, and on their arrangement and linkages. Various tissues have been taken to exemplify the mechanical behaviour of these wall constituents; the reported values of the elastic moduli of these tissues are presented in tabular form (Table 3). However, some caution is necessary when comparing these values with those found for arteries. In tissues such as the arterial wall the collagen forms, at moderate strains, a loose mesh. Only when fully distended, so that the collagen comes to lie more nearly parallel, would one expect the modulus of this material to approach that shown by the dense material which forms tendon. Ligamentum nuchae is known to contain some collagen. Hass (1942) extracted aortic collagen with formic acid and studied the properties of the remaining elastin, which were probably not entirely unchanged by this treatment. Burton (1954) has calculated the elastic modulus of elastin to be 3 × 10^6 dynes/cm^2 from Hass's data. This figure is based on the cross-sectional area of a desiccated specimen and should be nearer 1 × 10^6 (McDonald, 1960). It is

probable that the true value lies between this figure and that reported for ligamentum nuchae, which is 6.

Mammalian smooth muscle is an unsatisfactory material for mechanical studies, and molluscan unstriated muscle may well be considerably different from that found in mammalian blood vessels. Ducret (1931) estimated the maximal tension exerted by the muscle in a cat's mesenteric artery to be $0{\cdot}8 \times 10^6$ dynes/cm^2. This value is based on histological estimates of the amount of muscle present and cannot be very accurate. However, the elastic modulus of active muscle must be considerably greater than the tension it can exert.

TABLE 3. The elastic moduli (E) of arterial wall constituents

Tissue	E (dynes/cm$^2 \times 10^6$)	Author
Collagen (tendon)	100	Reuterwall (1921)*
	30–100	Krafka (1939)
	1000	Burton (1954) (30 % strain)
Elastin (ligamentum nuchae)	6	Reuterwall (1921)*
	6	Wöhlisch, de Rochement & Gerschler (1926)
	6	Krafka (1939)
Smooth muscle (resting)	0·1	Bozler (1936) (molluscan)
	0·1	Krafka (1939) (mammalian taenia coli)
	2·5	Reichel (1952)* (molluscan)

* Calculated by Bergel.

It is generally assumed that the collagen and elastin in the arterial wall function in parallel. This concept appears to have been first clearly formulated by Reuterwall (1921); it is supported by the histological studies of Benninghof (1930) and by the results reported here. Arterial moduli of the order of 20×10^6 dynes/cm^2 (see Table 1) can only result from the presence of some material as strong as collagen which is not in series with any weaker substance. The thoracic aorta contains about 1/6 collagen (Harkness, Harkness & McDonald, 1957); if this is arranged wholly circumferentially and bears all the load at full distension it is then exhibiting a modulus of 120×10^6 dynes/cm^2, which is similar to that reported for tendon by Reuterwall (1921) and Krafka (1939).

It is not easy to see why the carotid wall should have reached a limiting modulus of about 13×10^6 dynes/cm^2, for there is no sign that a similar limit has been approached in the other vessels. At a pressure of 220 mm Hg the tension on the collagen in the carotid wall is of the order of 10 kg/mm^2 and it is conceivable that the ground substance cementing the fibres might begin to flow. However, there was no sign that the vessels were in any way damaged by these pressures, which are not far outside the physiological range for the dog.

As the vessel is allowed to deflate the load is progressively passed from

the relatively inextensible collagen on to the elastin. It has been suggested that this tissue bears all the tension at pressures below 100–150 mm Hg (Reuterwall, 1921; Roach & Burton, 1957). However, the wall modulus of thoracic aorta at 100 mm Hg, $4{\cdot}4 \times 10^6$ dynes/cm^2, if due solely to the elastin present (30%, Harkness *et al.* 1957) would require the elastin to have a modulus twice as great as the values given in Table 3. Reuterwall's (1921) data show that the modulus of ligamentum nuchae at low strains is considerably smaller. It seems more likely, as McDonald (1960) has suggested, that even at the lowest pressures some material stronger than elastin, presumably collagen, contributes significantly to the arterial wall modulus. Elastin may be readily extended to 250% of its original length (Reuterwall, 1921) and it may be concluded that the difference in behaviour between the thoracic aorta and the other vessels between 60 and 100 mm Hg pressure (Fig. 1) is due to the great preponderance of elastin over collagen (2:1) that has been found (Harkness *et al.* 1957) only in the thoracic aorta, and to a relatively loose collagen network.

There is relatively little difference between the moduli of elastin and smooth muscle (Table 3) and similar arguments cannot be used to decide the disposition of these materials in the vessel wall. Histological observations (Benninghof, 1930) indicate that they are arranged in parallel, and this is supported by similar (unpublished) studies of my own. There can be no other explanation for the behaviour illustrated in Fig. 4 than that the material which was extended by a steady high pressure, presumably muscle, is arranged in parallel with the other elements. Since this specimen was rather stiffer during the first inflation (crosses) than subsequently it is possible to make an estimate of the modulus of resting smooth muscle. Assuming that the femoral wall contains 75% muscle, a value of about 1×10^6 dynes/cm^2 has been calculated. This figure is also based on the assumption that the stretched muscle subsequently develops no significant elastic tension (Abbott & Lowy, 1958); if this is not the case the figure should be higher. A similar value has been estimated from measurements of dynamic elasticity (Bergel, 1961). In view of these results, which are similar in value to those quoted in Table 3, the hypothesis of Burton (1954) that the muscle works through a complex system of linkages, conferring a mechanical advantage of about 100:1, is unnecessary. This suggestion stemmed from the assumption that the elastic modulus of smooth muscle was much lower, $0{\cdot}06 \times 10^6$ dynes/cm^2.

A parallel arrangement of the constituents implies that the properties of the arterial wall are more directly related to radius than to pressure, and this may be clearly seen in Fig. 4. For practical purposes it is more useful to relate the modulus to pressure, as has been done here. It should be remembered that, under normal conditions, arteries are prevented from

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As the vessel is allowed to deflate the load is progressively passed from

the relatively inextensible collagen on to the elastin. It has been suggested that this tissue bears all the tension at pressures below 100–150 mm Hg (Reuterwall, 1921; Roach & Burton, 1957). However, the wall modulus of thoracic aorta at 100 mm Hg, $4{\cdot}4 \times 10^6$ dynes/cm^2, if due solely to the elastin present (30%, Harkness *et al.* 1957) would require the elastin to have a modulus twice as great as the values given in Table 3. Reuterwall's (1921) data show that the modulus of ligamentum nuchae at low strains is considerably smaller. It seems more likely, as McDonald (1960) has suggested, that even at the lowest pressures some material stronger than elastin, presumably collagen, contributes significantly to the arterial wall modulus. Elastin may be readily extended to 250% of its original length (Reuterwall, 1921) and it may be concluded that the difference in behaviour between the thoracic aorta and the other vessels between 60 and 100 mm Hg pressure (Fig. 1) is due to the great preponderance of elastin over collagen (2:1) that has been found (Harkness *et al.* 1957) only in the thoracic aorta, and to a relatively loose collagen network.

There is relatively little difference between the moduli of elastin and smooth muscle (Table 3) and similar arguments cannot be used to decide the disposition of these materials in the vessel wall. Histological observations (Benninghof, 1930) indicate that they are arranged in parallel, and this is supported by similar (unpublished) studies of my own. There can be no other explanation for the behaviour illustrated in Fig. 4 than that the material which was extended by a steady high pressure, presumably muscle, is arranged in parallel with the other elements. Since this specimen was rather stiffer during the first inflation (crosses) than subsequently it is possible to make an estimate of the modulus of resting smooth muscle. Assuming that the femoral wall contains 75% muscle, a value of about 1×10^6 dynes/cm^2 has been calculated. This figure is also based on the assumption that the stretched muscle subsequently develops no significant elastic tension (Abbott & Lowy, 1958); if this is not the case the figure should be higher. A similar value has been estimated from measurements of dynamic elasticity (Bergel, 1961). In view of these results, which are similar in value to those quoted in Table 3, the hypothesis of Burton (1954) that the muscle works through a complex system of linkages, conferring a mechanical advantage of about 100:1, is unnecessary. This suggestion stemmed from the assumption that the elastic modulus of smooth muscle was much lower, $0{\cdot}06 \times 10^6$ dynes/cm^2.

A parallel arrangement of the constituents implies that the properties of the arterial wall are more directly related to radius than to pressure, and this may be clearly seen in Fig. 4. For practical purposes it is more useful to relate the modulus to pressure, as has been done here. It should be remembered that, under normal conditions, arteries are prevented from

collapsing fully. Nevertheless, it takes a considerable time under pressure for a collapsed vessel to regain its original size, and significant changes in arterial elasticity are to be expected immediately following any substantial fall in blood pressure.

The amount of retraction seen in these vessels is very similar to that reported by Fuchs (1900) sixty years ago. It can be seen from Fig. 2 how greatly changes in length can alter the behaviour of arteries quantitatively and qualitatively. This shortening should obviously be taken into account in any study of arterial elasticity, particularly when longitudinal strips are used.

SUMMARY

1. The use of a simple incremental modulus of elasticity is proposed for description of the mechanical properties of the arterial wall. Measurements of this modulus were made on the thoracic and abdominal aorta, femoral and carotid arteries, of the dog. In all these experiments the vessel was held at the length it had in life.

2. The arterial wall becomes stiffer as it is extended. This increase is less marked in the thoracic aorta up to a pressure of 100 mm Hg. Mean values for the static elastic modulus (dynes/cm^2 $\times$ 10^6) at 100 mm Hg pressure were thoracic aorta 4·3, abdominal aorta 8·7, femoral artery 6·9, carotid artery 6·4.

3. The elastin, collagen and smooth muscle in the arterial wall are arranged in parallel and each bears some load at all internal pressures.

4. The elastic modulus of resting vascular smooth muscle is probably in the region of 1×10^6 dynes/cm^2.

5. Values for the relative wall thickness and the retraction on excision are given.

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REFERENCES

Abbott, B. C. & Lowy, J. (1958). Mechanical properties of *Helix* and *Mytilus* muscle. *J. Physiol.* **141**, 398–407.

Benninghof, A. (1930). Blutgefässe und Herz. In *Handbuch der Mikroskopischen Anatomie des Menschen*. Berlin: Springer.

Bergel, D. H. (1958). A photo-electric method for the determination of the elasto-viscous behaviour of the arterial wall. *J. Physiol.* **141**, 22–23*P*.

Bergel, D. H. (1960). *The visco-elastic properties of the arterial wall*. Ph.D. thesis, University of London.

Bergel, D. H. (1961). The dynamic elastic properties of the arterial wall. *J. Physiol.* (in the Press).

Bozler, E. (1936). An analysis of the properties of smooth muscle. *Cold Spr. Harb. Symp. quant. Biol.* **4**, 260–266.

BURTON, A. C. (1954). Relation of structure to function of the tissues of the wall of blood vessels. *Physiol. Rev.* **34**, 619–642.

DUCRET, S. (1931). Rhythmische Tonusschwankungen und Adrenalinerregbarkeit der mesenterial Gefässe. *Pflüg. Arch. ges. Physiol.* **227**, 753–758.

FENN, W. O. (1957). Changes in length of blood vessels on inflation. In *Tissue Elasticity*, ed. REMINGTON, J. R. Washington: American Physiological Society.

FUCHS, R. F. (1900). Zur Physiologie und Wachstumsmechanik des Blutgefäss-systems. *Arch. Anat. Physiol.* (1900) 102–154.

HALLOCK, F. & BENSON, I. C. (1937). Studies on the elastic properties of human isolated aorta. *J. clin. Invest.* **16**, 595–602.

HARDUNG, V. (1953). Vergleichende Messungen der dynamische Elastizität und Viskosität Blutgefässen, Kautschuk und synthetischen Elastomeren. *Helv. physiol. acta*, **11**, 194–211.

HARKNESS, M. L. R., HARKNESS, R. D. & MCDONALD, D. A. (1957). The collagen and elastin content of the arterial wall in the dog. *Proc. Roy. Soc.* B, **146**, 541–551.

HASS, G. M. (1942). Elastic tissue 2. A study of the elasticity and tensile strength of elastic tissue isolated from the human aorta. *Arch. Path. (Lab. Med.)*, **34**, 971–981.

KING, A. L. & LAWTON, R. W. (1950). Elasticity of body tissues. In *Medical Physics*, **2**, ed. GLASSER, O. Chicago: Year Book Publishers.

KRAFKA, J. (1939). Comparative studies of the histo-physics of the aorta. *Amer. J. Physiol.* **125**, 1–14.

LAMBOSSY, P. & MÜLLER, A. (1954). Vitesse des ondes dans les tubes. Influence de l'anisotropie de la paroi. *Helv. physiol. acta*, **12**, 217–229.

LAWTON, R. W. (1954). The thermoelastic behaviour of isolated aortic strips of the dog. *Circulation Res.* **2**, 344–353.

LAWTON, R. W. (1955). Measurement of the elasticity and damping of isolated aortic strips of the dog. *Circulation Res.* **3**, 403–408.

LAWTON, R. W. (1960). Variability of the viscoelastic constants along the aortic axis of the dog. *Circulation Res.* **8**, 381–389.

LOVE, A. E. H. (1927). *A Treatise on Mathematical Elasticity*, 3rd ed. Cambridge University Press.

MCDONALD, D. A. (1960). *Blood Flow in Arteries*. London: Arnold.

REICHEL, H. (1952). Die elastischen Eigenschaften des glatten Schliessmuskeln von Pinna Nobilis bei verscheidenen Tonuslängen unter statischen und dynamischen Bedingungen. *Z. Biol.* **105**, 162–169.

REMINGTON, J. W. (1955). Hysteresis loop behaviour of the aorta and other extensible tissues. *Amer. J. Physiol.* **180**, 83–95.

REUTERWALL, O. P. (1921). Über die Elastizität der Gefässwände und die Methoden ihrer näheren Prüfung. *Acta med. scand.* Suppl. 2, 1–175.

ROACH, M. R. & BURTON, A. C. (1957). The reason for the shape of the distensibility curves of arteries. *Canad. J. Biochem. Physiol.* **35**, 681–690.

ROY, C. S. (1880). The elastic properties of the arterial wall. *J. Physiol.* **3**, 125–162.

WAGNER, R. & KAPAL, E. (1951). Über Eigenschaften des Aortenwindkessels. I. *Z. Biol.* **104**, 169–202.

WÖHLISCH, E., DE ROCHEMENT, R. DU M. & GERSCHLER, H. (1926). Untersuchungen über die elastischen Eigenschaften tierischer Gewebe. *Z. Biol.* **85**, 324–341.

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THE DYNAMIC ELASTIC PROPERTIES OF THE ARTERIAL WALL

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Although the response of the arterial tree to relatively slow changes in blood pressure is determined by its static elastic properties (Bergel, 1961), the rapid pressure changes occurring at each heart beat will result in rather different behaviour. This is due to the visco-elastic properties of the arterial wall. The mechanical response of a visco-elastic material depends both on the force applied (elastic response) and on the time it acts (viscous response). These substances display 'creep' (continuing extension at constant load) and stress relaxation (tension decay at constant length). The properties of such a material can be defined by measurements of either of these phenomena, but it is difficult to infer from this the response to a rapid stress. A more direct form of enquiry is to measure the oscillatory stress–strain relationships at any required frequency, which has previously been done with strips and rings cut from arteries and recently with intact vessels *in vivo* (Petersen, Jensen & Parnell, 1960). When using intact vessels it is necessary to measure simultaneously the amplitude and the phase relationships of oscillatory pressure and radius changes. As arteries change very little in length with each heart beat (Lawton & Greene, 1956) they should be held at their natural length.

METHODS

The apparatus used is shown diagrammatically in Fig. 1; its use has been explained previously (Bergel, 1961). Changes in the radius of the specimen are detected with a collimated light beam and a photomultiplier tube (R.C.A. 931A).

Sinusoidal variations in volume were superimposed upon that due to a steady pressure of 100 mm Hg by a cam-operated pump (Taylor, 1959) leading to the vessel through the lower of the two supports. The pressure changes developed were detected by a capacitance manometer (Southern Instruments) attached to a wide cannula. This was inserted through the upper support and its tip lay in the vessel at the level where radius changes were detected by the light beam.

To determine the amplitude and phase distortion the manometer was calibrated dynamically, both by the pressure transient method of Hansen (1949) and by direct measurement. The latter was achieved by comparing its output at frequencies of 2–25 c/s with that from an air-filled unit with a natural frequency of 940 c/s. The manometer and cannula used in

these experiments was found to have an undamped natural frequency of 89 c/s and a degree of damping of 0·102. According to Hansen's formulae it should, at 20 c/s, over-register pressure changes by 5·2% with a phase lag of 0·048 radians. This was confirmed, within the limits of error, by the direct measurements. Accordingly the appropriate corrections were made to all readings.

Automatic Fourier analysis of the signals representing pressure and radius changes was performed by a resolver (Taylor, 1959), synchronized by a switch-plate mounted on the cam-shaft of the pump. Direct readings of the sine and cosine coefficients of both variables could be made at each frequency.

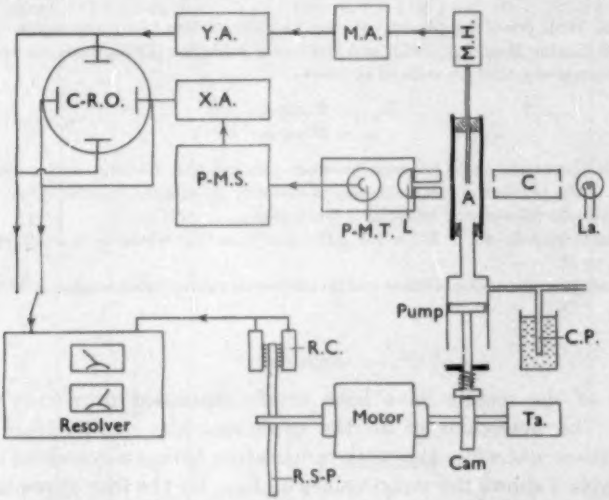

Fig. 1. Formalized diagram of the apparatus. A, arterial specimen; C, collimating tube; La, light source; C.P., mercury-column manostat; Ta, tachometer; R.S-P., resolver switch-plate; R.C., resolver contacts; L, lens; P-M.T., photomultiplier tube; P-M.S. photomultiplier power supply; M.H., manometer transducer head; M.A. manometer amplifier; X.A., X amplifier; Y.A., Y amplifier; C-R.O., cathode-ray oscilloscope.

Dynamic measurements were normally made after determining the static stress–strain relationships (Bergel, 1961) but in some cases the dynamic experiments alone were performed. These vessels were held at a steady pressure of 100 mm Hg for some minutes to allow stress relaxation.

The specimens used were dog's thoracic and abdominal aorta, femoral and carotid arteries, removed and studied within a few hours of death by bleeding. These were filled with isotonic saline at room temperature. The frequency range covered was from 2 to 18 c/s.

The markedly non-linear properties of the arterial wall (Bergel, 1961) lead to two difficulties. In the first place, a fall in mean pressure due to a small leak will result in a diminished elastic modulus. As small a change as 2 mm Hg could be detected in this way. To overcome this the barrel of the pump was connected, by a narrow tube, to a saline reservoir held at a constant pressure of 100 mm Hg by a mercury column manostat (Fig. 1, C.P.). This also altered the magnitude and phase of the pressure changes, but since the radius changes were similarly affected (as was shown by direct experiment) no errors resulted.

Secondly, the non-linearity of the wall will distort the pressure changes generated by a sinusoidal alteration in volume. However, provided the oscillations are kept small enough, this distortion is negligible. The appearance or distortion could be observed on the monitor oscilloscope, and the amount of second and third harmonic generated could also be measured by the resolver. The pressure changes used were ±5–10 mm Hg and the distortion was insignificant. This represents radius changes of less than ±2–3 %.

The dynamic elastic modulus (E') is given by

$$E' = \frac{\Delta P}{\Delta R_o} \frac{2(1-\sigma^2)R_o R_i^2}{R_o^2 - R_i^2}$$

(see Bergel, 1961, preceding paper). E' may be resolved into two components, elastic and viscous. Following Hardung (1953) and McDonald & Taylor (1959) these are termed E_{dyn} and $\eta\omega$ respectively, and are defined as follows:

$$E_{dyn} = E' \cos \phi,$$
$$\eta\omega = E' \sin \phi,$$

where ϕ is the phase angle between pressure changes (ΔP leading) and radius changes (ΔR_o), $\eta\omega$ is the product of the coefficient of viscosity (η) and the angular velocity (ω), but only in a simple visco-elastic material is η constant.

Thus the amplitude of E', $|E'| = [(E_{dyn})^2 + (\eta\omega)^2]^{\frac{1}{2}}$, so that where $\eta\omega$ is small, say $< 10\,\%$ E_{dyn}, $E_{dyn} \simeq E'$.

Repeated determinations of these two quantities on rubber tubes suggest an over-all error of ±5 %.

RESULTS

Some of the results have been briefly discussed previously (Bergel, 1960*a*). The behaviour of all the specimens was qualitatively similar, though there was wide quantitative variation between vessels of the same type. Table 1 shows the mean values of E_{dyn} for the four types of artery. It will be seen that all vessels showed an abrupt increase in stiffness between frequencies of 0 (i.e. static) and 2 c/s; with increasing frequency there was little further change. This increase appears to be greater for the more muscular vessels. This is well seen when the modulus ratio (E_{dyn}/E_{static}) is plotted against frequency (Fig. 2). This ratio correlates well with the muscularity of the vessel, though the variation is so great as to render the differences rarely significant (the vertical lines indicate the standard errors).

In all cases the viscous component ($\eta\omega$) was relatively small and again the relation to frequency was similar in all. At 2 c/s $\eta\omega$ was about 0·1 E_{dyn} and it increased by only about 1·5–2 times at 18 c/s. Since $\eta\omega = E' \sin \phi$ its magnitude depends on that of the complex modulus itself. This can be seen in Fig. 3 (top half) where it appears that the size of $\eta\omega$ also depends on the muscularity of the wall. However, the relation between E_{dyn} and $\eta\omega$ was remarkably similar for all specimens. Since $\phi = \tan^{-1} \eta\omega/E_{dyn}$ the variation of ϕ with frequency has been plotted in the lower half of Fig. 3. It can also be seen in this figure that the phase angle was very

TABLE 1. Mean values of E_{dyn} (dynes/cm^2 ± S.E. of means)
(Figures in brackets give the number of specimens.)

Vessel	0 c/s	2 c/s	5 c/s	18 c/s
Thoracic aorta	4·4 ± 0·40 (10)	4·7 ± 0·42 (10)	4·9 ± 0·45 (10)	5·3 ± 0·80 (4)
Abdominal aorta	9·2 ± 0·94 (7)	10·9 ± 0·88 (7)	11·0 ± 0·82 (7)	12·2 ± 0·46 (4)
Femoral artery	9·0 ± 1·15 (5)	12·0 ± 0·81 (5)	12·0 ± 0·82 (5)	10·6 ± 1·39 (5)
Carotid artery	6·9 ± 0·48 (6)	11·0 ± 1·00 (6)	11·3 ± 0·99 (6)	11·5 ± 1·03 (6)

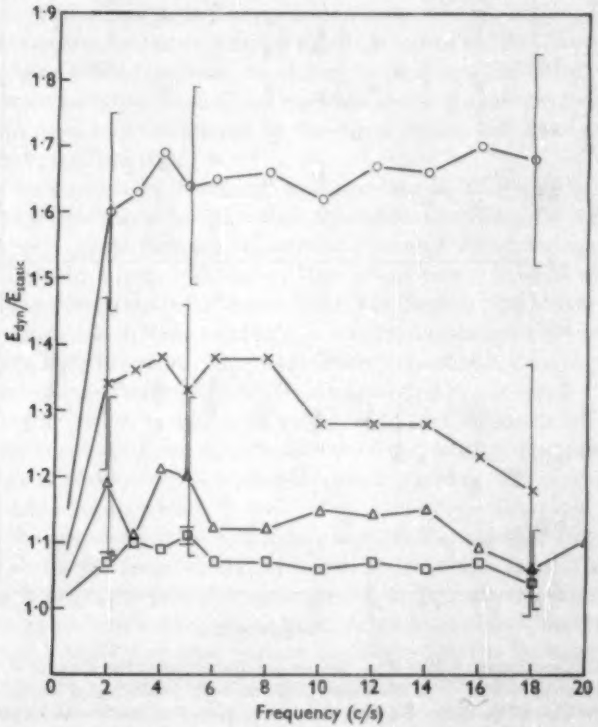

Fig. 2. Mean values for the relative modulus (E_{dyn}/E_{static}). Note the sharp increase between 0 and 2 c/s which correlates with the amount of smooth muscle in the vessel wall. The vertical lines show the standard errors. p values for the differences seen are as follows:

Difference between	2 c/s	5 c/s	18 c/s
Thoracic and abdominal	0·1	0·15	0·35
Abdominal and femoral	0·23	0·13	0·35
Femoral and carotid	0·1	0·1	0·05

□ thoracic aorta; △ abdominal aorta; × femoral artery; ○ carotid artery.

small (5–10°) and it could well have been overlooked entirely by merely inspecting the records of pressure and radius.

There was no difference in ϕ between different vessels at frequencies above 5 c/s, but at the lowest frequency it was rather smaller for the aortic specimens than for the other, more muscular, vessels. If the means

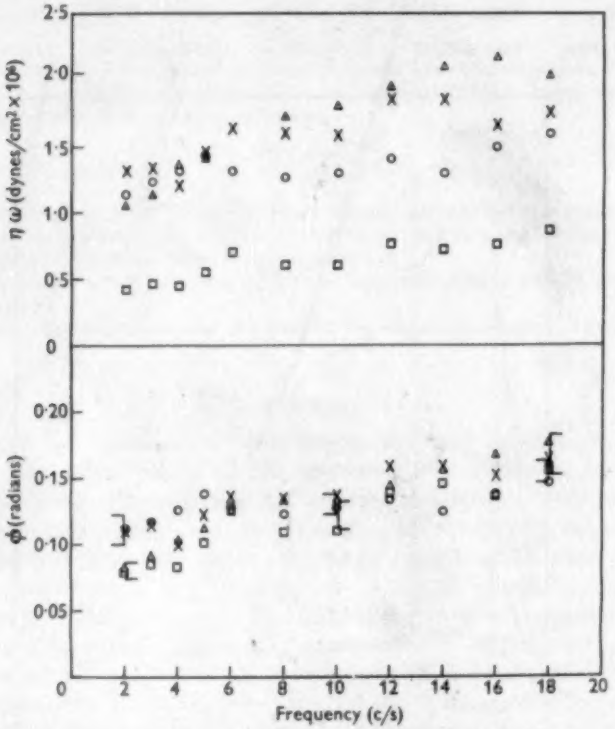

Fig. 3. Above: mean values of $\eta\omega$, the viscous component of the dynamic modulus. Below: mean values of ϕ, the phase angle between pressure and displacement. Note the great similarity between all vessels, except at the lowest frequencies, and the small change in ϕ between 5 and 18 c/s. The vertical lines show S.E. of means for the grouped means of the thoracic and abdominal aorta and the femoral and carotid arteries. □ thoracic aorta; △ abdominal aorta; × femoral artery; ○ carotid artery.

and standard errors for these two groups of artery are computed, a significant ($0{\cdot}02 > P > 0{\cdot}01$) difference appears between them at 2 c/s only. The standard errors for the two groups are shown in Fig. 3 (bottom half).

A few measurements were made at mean pressures of 50 and 150 mm Hg. The scatter was wide, but it appeared that the modulus ratio was independent of mean strain.

DISCUSSION

These results differ in some respects from those previously reported. Hardung (1953), studying aortic strips, found a modulus ratio of 1·25 at 2 c/s which did not alter up to 15 c/s. At low frequencies $\eta\omega$ was about 0·1 E_{dyn}, but thereafter it rose linearly with increasing rate of strain. Kapal (1954) with a similar technique and using human specimens, reported that the ratio increased steadily to 1·3 at 40 c/s (mean of 22). No measurements of phase differences were made but inspection of his records shows that they were no larger than those reported here. Kapal also found that the modulus ratio was not altered by the mean strain, but Lawton (1955) was unable to confirm this.

The rise in $\eta\omega$ seen by Hardung, and increase in E' found by Kapal, may be due to the use of strips rather than intact vessels. Ranke (1934) reported a very great increase in arterial viscosity when the specimens were immersed in hypertonic saline. This effect was reversible and was presumably due to removal of water from the tissues. The whole vessels filled with saline which were used in this work were always kept moist by seepage from tiny branches, but it is clearly considerably more difficult to maintain constant hydration of strips suspended in air.

A different approach is due to Lawton (1955, 1960) who has calculated the dynamic modulus from measurements of the natural frequency and rate of decay of oscillations in loaded longitudinal strips. These specimens were suspended in air at 25° C and 100% humidity. Lawton's results show that the static modulus and modulus ratio were similar (*ca.* 2×10^6 dynes/cm^2 and 2–2·5 respectively) in the aorta, iliac and carotid arteries of the dog, though the modulus ratio tended to be rather greater in the carotid. The rather low moduli might be expected from somewhat retracted strips (Bergel, 1961) but this cannot acccount for the similar ratios. Lawton (personal communication) has suggested that this might be due to the storage of his specimens in an ice-box for 24–36 hr before the experiment. It will be seen that the modulus ratio probably depends on the amount of muscle in the artery, a tissue which would be very sensitive to differences in experimental method.

Measurements made *in vivo* of the relationship between arterial pressure and diameter have been reported recently (Peterson *et al.* 1960). The dynamic modulus was found to be essentially constant over a range of about 2–10 c/s with values rather greater than those reported here. The viscous modulus was very much smaller, though it is not clear to what extent

it varied with frequency. There was a small phase difference between pressure and dilatation, with a delay usually less than 5 msec, though the authors were not very confident of the accuracy of determination of these very short times. This delay would represent a phase lag of 0·06–0·3 radians, very similar to that found here *in vitro*. Muscular activity has been shown both by Peterson *et al.* (1960) and by Hardung & Laszt (1960) to increase both the viscous and elastic components of the dynamic modulus and this may account, partly at least, for the rather high modulus measured *in vivo*. The excised specimens considered in this paper showed occasional spontaneous muscular contractions but it is likely that the over-all level of tone was very low. Nevertheless, it will be seen that the values reported here predict reasonable values for the pulse-wave velocity in the vessels concerned, while the results of Peterson *et al.* suggest velocities considerably higher than those generally accepted.

The behaviour seen in the arteries is not to be expected from any simple mathematical model embodying only viscous and elastic properties and not those due to inertia. Two lines of evidence suggest that this omission is reasonable. First, the computed resonant frequency for the thoracic aorta would not be lower than 300 c/s, very much higher than the frequencies actually employed. In the case of the other vessels the resonant frequency must be considerably higher. Secondly, inertial effects may be entirely discounted in the arterial stress-relation experiments of Zatzman Stacy, Randall & Eberstein (1954). A digital computer has been used to calculate the dynamic response of a blood vessel in which the pressure decayed in the manner found by these workers. Within the limits of the data fed to the computer the predicted behaviour fitted well with that actually observed.

Attempts were therefore made to fit the observed properties to those shown by a number of simple models composed solely of viscous and elastic elements in various combinations. These attempts were not successful. Possibly a much more complex model could be devised which would prove suitable, but the biological realities underlying this would not be readily apparent. This applies particularly to the concept of muscle viscosity and it is doubtful whether at the present time the construction of complex mathematical models of this sort serves any useful purpose.

The main conclusion to be drawn from Fig. 2 is that the modulus ratio (E_{dyn}/E_{static}) depends on the amount of muscle in the arterial wall (since $\eta\omega = ca.\ 0{\cdot}1\ E_{dyn}$ there is little error in taking E_{dyn} as the dynamic modulus). Figure 3 further suggests that the viscous component in all these vessels is of a similar nature, and that it too is related to the smooth muscle.

In Figure 2 it can be seen that there is a relatively narrow frequency range in which the elastic modulus changes from the static to the dynamic value. This is known as the critical frequency, and for all arteries it is clearly below 2 c/s. Some experiments of Hardung (1953) at very low frequencies suggest it lies below 0·5 c/s. The critical frequency for resting molluscan smooth muscle has been determined (Pryor, 1952) and found to be below 0·02 c/s. This indicates that smooth muscle will show relatively long time constants for creep and stress relaxation and it has been reported by Abbot & Lowy (1957) that after a passive stretch tension decay in molluscan unstriated muscle was a double exponential with time constants of 5·8 and 68 sec. It may be concluded that the dynamic properties of arteries are dominated by the behaviour of smooth muscle but that the properties of muscle are too complex to be characterized by a simple spring-dashpot model.

If the change in modulus over the critical range is due to the inability of a smooth-muscle component to respond to rapid changes in tension it should be possible to estimate the contribution of the muscle to the modulus of the arterial wall. From data given by Reichel (1952) one may calculate values of 2·5 and $4{\cdot}5 \times 10^6$ dynes/cm^2 for the static and dynamic moduli respectively of resting molluscan muscle, i.e. a relative modulus of about 2. Assuming that this is the case for vascular smooth muscle, and that the femoral artery contains about 75% smooth muscle, and the carotid about 90% (unpublished observations) it appears that resting smooth muscle in the arterial wall has a static elastic modulus of $1\text{–}1{\cdot}5 \times 10^6$ dynes/cm^2. This calculation cannot be done for the aorta owing to the more complex structure of that vessel. The many assumptions made may well be in error, but this figure is in the range of values quoted in the literature for various types of unstriated muscle and a similar figure was suggested by static inflation experiments (Bergel, 1961).

The predicted pulse-wave velocity (p.w.v.)

The velocity of propagation of a pressure wave (m/sec) in a fluid-filled, thin-walled elastic tube is given by the Moens–Korteweg equation:

$$c = (Eh/2R\rho)^{\frac{1}{2}},$$

where E is the elastic modulus of the tube wall (dynes/cm^2),

ρ is the density of the fluid,

$h/2R$ is the relative wall thickness, the ratio of wall thickness (h) to the mean diameter ($2R$).

The relative wall thickness is somewhat greater in the smaller vessels (Bergel, 1961) but this increase is not enough, by itself, to account for the greater p.w.v. in these arteries.

This equation describes the behaviour of waves in a very thin-walled elastic tube filled with an incompressible non-viscous fluid, the radius being assumed to be very small compared with the wave-length. A full account of these factors is given by Lambossy (1950, 1951). The errors introduced by this last assumption are mostly very small, but the nature of the tube wall and the fluid viscosity are of more importance. The wall has been assumed to be an elastic boundary which increases in circumference alone when the pressure is raised, in effect taking the Poisson's ratio as zero. A further slight error is introduced by using the ratio of wall thickness to external radius rather than to the mean radius. An expression for the ratio of the true p.w.v. (c) to that obtained from the simple Moens–Korteweg equation (c'), in a tube of unaltering length can be derived (Bergel, 1960*b*). It is found that

$$\frac{c}{c'} = \left[\frac{2-\gamma,}{2-2\gamma(1-\sigma-2\sigma^2)+\gamma^2(1-\sigma-2\sigma^2)-2\sigma^2}\right]^{\frac{1}{2}},$$

where $\gamma = h/R_0$. It can be seen that when the wall is infinitely thin and $\sigma = 0$, $c = c'$. For arteries where $\gamma = 0{\cdot}1$–$0{\cdot}13$ and $\sigma = 0{\cdot}5$ the p.w.v. will be increased by about 12%.

Wave propagation in a viscous fluid has been studied by Womersley (1957*a*) and the influence of wall viscosity on phase velocity and damping was discussed by Taylor (1959). Using their equations and employing the experimentally determined values for wall thickness and dynamic modulus, the p.w.v. and damping in these vessels have been computed. It is also assumed that the arteries are very stiffly tethered and loaded (Womersley, 1957*b*). This implies that they are unable to move longitudinally under the influence of the viscous drag from the blood flow. There is experimental evidence (Lawton & Greene, 1956) that the longitudinal movements of the wall are extremely small, though an untethered tube might be expected to show considerable movements (McDonald, 1960).

Figure 4 shows the computed values for the phase velocity and for the transmission (fraction of wave amplitude transmitted) over a distance of 10 cm. In the presence of reflexions the phase velocity can be considerably altered but this effect will be less marked for high-frequency, short wave-length oscillations (McDonald & Taylor, 1959). The foot-to-foot p.w.v. is that of the high-frequency components responsible for the sharp inflexion at the foot of the pulse wave and should therefore be compared with the phase velocity predicted for the higher frequencies.

Measured pulse-wave velocities have been fully listed previously (Bergel, 1960*b*). The following figures give the range of the reported values: thoracic aorta, 4·5–7 m/sec; abdominal aorta, 5·5–8·5 m/sec; femoral artery, 8–12 m/sec. Though no measurements of carotid p.w.v. have been

reported, the *in vitro* experiments of Bramwell, Downing & Hill (1923) suggest that it is similar to that in the femoral.

The calculated figures are in good agreement with those quoted. The predicted velocity in the abdominal aorta is high and that for the femoral

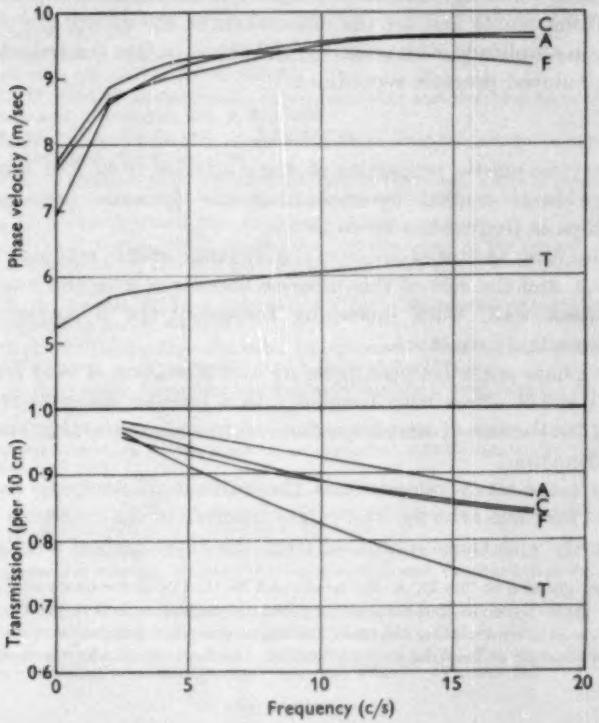

Fig. 4. Above: variation of phase velocity with frequency. Below: variation of wave transmission (amplitude of downstream oscillations/amplitude of upstream oscillations) over a distance of 10 cm. All these curves are computed from the experimental results. The letter at the end of each curve indicates the type of artery. The range of reported pulse-wave velocities is as follows: thoracic aorta 4·5–7 m/sec, abdominal aorta 5·5–8·5 m/sec, femoral artery 8–12 m/sec.

and carotid arteries lies at the bottom of the range, but it has been shown that muscular activity will increase the velocity by increasing both E_{dyn} and $\eta\omega$ (Hardung & Laszt, 1960; Peterson *et al.* 1960). There is little direct evidence on the rate of damping of the pulse wave, which cannot be measured from changes in amplitude if reflexions are present. The transmission is very low in the thoracic aorta, for in this vessel 10 cm is a

considerable fraction of the high-frequency wave-length. Certainly the sharp dicrotic notch seen in recordings from this vessel is damped out very rapidly. Landowne (1957) has studied the transmission of superimposed pressure spikes in human brachial arteries; in this case reflexions have no effect. Taking reasonable values for the distance separating his two recording points and for the dimensions of the artery the predicted reduction in amplitude is about 60 %, and this is in fact the reduction seen in the reproduced pressure recordings.

SUMMARY

1. The visco-elastic properties of dog's arteries from four anatomical sites have been studied by measuring the dynamic pressure–radius relationships at frequencies up to 20 c/s.

2. There is an abrupt increase in the dynamic elastic modulus between 0 and 2 c/s, and the size of this increase correlates with the muscularity of the vessel wall. With increasing frequency the dynamic modulus remains essentially constant.

3. The phase angle between pressure and dilatation is very small (less than 10°) and it alters with frequency in a manner different from that predicted by the use of simple mechanical models containing elastic and viscous elements.

4. The pulse-wave velocity and the amount of damping have been calculated for these arteries. The values reported in the literature compare satisfactorily with those computed from the experimental results.

I am very grateful to Drs D. A. McDonald and M. G. Taylor for much advice and discussion, to Mr D. C. Moore and his staff for technical assistance, and to the Royal Society for the provision of a calculating machine. The digital computer calculations were performed by Dr S. Michaelson of Imperial College, London, to whom my thanks are also due.

REFERENCES

Abbott, B. C. & Lowy, J. (1957). Stress relaxation in muscle. *Proc. Roy. Soc.* B, **146**, 281–288.

Bergel, D. H. (1960*a*). The dynamic elastic modulus of the arterial wall. *J. Physiol.* **151**, 26–27*P*.

Bergel, D. H. (1960*b*). *The visco-elastic properties of the arterial wall.* Ph.D. thesis, University of London.

Bergel, D. H. (1961). The static elastic properties of the arterial wall. *J. Physiol.* (in the Press).

Bramwell, J. C., Downing, A. C. & Hill, A. V. (1923). The effect of blood pressure on the extensibility of the human artery. *Heart*, **10**, 289–300.

Hansen, A. T. (1949). Pressure measurement in the human organism. *Acta physiol. scand.* **19**, Suppl. 68.

Hardung, V. (1953). Vergleichende Messungen der dynamischen Elastizität und Viskosität von Blutgefässen, Kautschuk und synthetischen Elastomeren. *Helv. physiol. acta*, **11**, 194–211.

Hardung, V. & Laszt, L. (1960). Visco-elastic behaviour of blood vessels of the muscular type. *Nature, Lond.*, **187**, 330–331.

KAPAL, E. (1954). Die elastischen Eigenschaften der Aortenwand sowie des elastischen kollagenen Bindegewebes bei frequenten zyklischen Beanspruchungen. *Z. Biol.* **107**, 347–404.

LAMBOSSY, P. (1950). Aperçu historique et critique sur le problème de la propagation des ondes dans un liquide compressible enfermé dans un tube élastique. Partie 1. *Helv. physiol. acta*, **8**, 209–227.

LAMBOSSY, P. (1951). Aperçu historique et critique sur le problème de la propagation des ondes dans un liquide compressible enfermé dans un tube élastique. Partie 2. *Helv. physiol. acta*, **9**, 145–161.

LANDOWNE, M. (1957). A method using induced waves to study pressure propagation in human arteries. *Circulation Res.* **5**, 594–601.

LAWTON, R. W. (1955). Measurements on the elasticity and damping of isolated aortic strips in the dog. *Circulation Res.* **3**, 403–408.

LAWTON, R. W. (1960). Variability of the viscoelastic constants along the aortic axis of the dog. *Circulation Res.* **8**, 381–389.

LAWTON, R. W. & GREENE, L. C. (1956). A method for the *in situ* study of aortic elasticity in the dog. *U.S. Naval Air Development Centre Report* No. NADC-MA-5603.

MCDONALD, D. A. (1960). *Blood Flow in Arteries.* London: Arnold.

MCDONALD, D. A. & TAYLOR, M. G. (1959). The hydrodynamics of the arterial circulation. *Progress in Biophysics*, **9**, 105–173, ed. BUTLER, J. A. V. & KATZ, B. London: Pergamon Press.

PETERSON, L. H., JENSEN, R. E. & PARNELL, J. (1960). Mechanical properties of arteries *in vivo*. *Circulation Res.* **8**, 622–639.

PRYOR, M. G. (1952). The rheology of muscle. In *Deformation and Flow in Biological Systems*, ed. FREY-WYSSLING, A. Amsterdam: North Holland Publishing.

RANKE, O. F. (1934). Die Dämpfung der Pulswelle und die innere Reibung der Arterienwand. *Z. Biol.* **95**, 179–204.

REICHEL, H. (1952). Die elastischen Eigenschaften des glatten Schliessmuskeln von Pinna nobilis bei verschiedenen Tonuslängen unter statischen und dynamischen Bedingungen. *Z. Biol.* **105**, 162–169.

TAYLOR, M. G. (1959). An experimental determination of the propagation of fluid oscillations in a tube with a visco-elastic wall; together with an analysis of the characteristics required in an electrical analogue. *Phys. Med. Biol.* **4**, 63–82.

WOMERSLEY, J. R. (1957*a*). An elastic tube theory of pulse transmission and oscillatory flow in mammalian arteries. *Wright Air Development Center, Technical Report* No. WADC-TR-56-614.

WOMERSLEY, J. R. (1957*b*). Oscillatory flow in arteries; the constrained elastic tube as a model of arterial flow and pulse transmission. *Phys. Med. Biol.* **3**, 178–187.

ZATZMAN, M., STACY, R. W., RANDALL, J. & EBERSTEIN, A. (1954). The time course of stress relaxation in isolated arterial segments. *Amer. J. Physiol.* **177**, 299–302.

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SOME EFFECTS OF STIMULATION OF THE MUSCLE NERVE ON AFFERENT ENDINGS OF MUSCLE SPINDLES, AND THE CLASSIFICATION OF THEIR RESPONSES INTO TYPES A1 AND A2

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The afferent discharge from single sense endings in mammalian muscle was first recorded by B. H. C. Matthews (1933). He was immediately faced with the problem of deducing the nature of any sense ending studied from the patterns of discharge in its afferent fibre. He found two distinct patterns of behaviour during contraction of the muscle elicited by weak stimulation of the mucle nerve, when the rate of discharge of some endings slowed (type A endings), while that of others accelerated (type B endings). Matthews concluded that the A endings were in the muscle spindles, and that the B endings were Golgi tendon organs. This classification has been generally accepted by later workers (Hunt & Kuffler, 1951*b*; Granit, 1955), and has been strengthened by the finding that only the A endings are affected by stimulation of the gamma efferents (Kuffler, Hunt & Quilliam, 1951; Hunt, 1954). Matthews in addition subdivided the A endings into types A 1 and A 2, and suggested that these corresponded to the flower-spray (A 1) and annulo-spiral (A 2) endings of the muscle spindle. The discharge of the A 1 endings slowed during muscle contraction, whether twitch or tetanic, under all the experimental conditions studied. In contrast, the discharge of the A 2 endings, though slowed during a submaximal contraction of the muscle, was dramatically accelerated when the stimulus to the nerve was increased, an effect which was attributed to excitation of the motor nerves to the intrafusal fibres of the muscle spindle. Thus it was suggested that contraction of the intrafusal muscle fibres produced strikingly different effects on the primary (annulo-spiral) and secondary (flower-spray) endings of the muscle spindle, a conclusion which is favoured by the different arrangement of these two types of endings within the muscle spindle.

More recent work, however, has cast doubt on the physiological significance of the subdivision of muscle spindle endings into types A 1 and A 2.

* M.R.C. Scholar.

The classification of an ending as a primary or secondary ending may now be made independently of its classification into type A 1 and A 2, by measuring the conduction velocity of its afferent fibre. For histological observations show that the axons forming the primary ending are of greater diameter, measured near the spindle, than those forming the secondary endings (Ruffini, 1898; Barker, 1948), while electrophysiological experiments show that in the muscle nerve the conduction velocities of the afferent fibres of muscle spindles range from 20 to 120 m/sec (Merton, 1953; Hunt, 1954), and that the reflex connexions of the smaller and larger of these afferent fibres are strikingly different (Eccles, Eccles & Lundberg, 1957; Eccles & Lundberg, 1959). It is therefore widely accepted that afferent fibres with a high conduction velocity come from primary endings and those with a low conduction velocity from secondary endings; but in terms of fibre conduction velocity the precise position and the sharpness of the division between afferents from primary and from secondary endings is not well established, though it is probably about 70 m/sec (Hunt, 1954). On the basis of this classification it has been found that stimulation of single gamma motor fibres, which supply solely the intrafusal fibres, can excite secondary as well as primary endings (Hunt, 1954; Kuffler *et al.* 1951). In addition the A 2 type of discharge is said to have been found both for primary (Hunt & Kuffler, 1951*b*) and for secondary endings (Hunt, 1954) on stimulating only large or alpha motor fibres. From all this work the A 2 type of discharge has been attributed not to activation of intrafusal muscle fibres but to the chance pull of extrafusal muscle fibres upon certain muscle spindles, and the difference between the A 1 and A 2 types of discharge has been considered to be fortuitous (Hunt & Perl, 1960).

There appears, however, to be a difference between the method used by Matthews to classify A 1 and A 2 endings and that used by Hunt and by Kuffler. Hunt & Kuffler (1951*a*, *b*) studied the behaviour of the spindle mainly during twitch contractions or during brief tetanic contractions in which the tension in the muscle barely reached a steady level; and in many cases they stimulated a ventral root filament rather than the muscle nerve. Inequalities in the rate or strength of contraction of different parts of the muscle might then well lead to the mechanical stimulation of some muscle spindles by chance pulls, and in conformity with this it may be noted that they recorded discharges of such varied patterns that they found it difficult to classify the endings into two clear groups. Matthews, on the other hand, stimulated only the whole muscle nerve and apparently relied mainly on the behaviour of the spindle during fairly prolonged tetanic contractions, for he showed that the behaviour of an A 2 ending during a twitch depended greatly upon the initial tension (see his Fig. 14); moreover, he found that almost all endings could be sharply classified. In

any case Matthews stated that he frequently found an increase in the discharge of the A 2 ending on increasing the strength of a stimulus which was already eliciting a maximal tetanic contraction of the muscle (see particularly his Fig. 13), and this cannot readily be attributed to the pull of extrafusal muscle fibres on the spindle. It may be doubted, therefore, whether Hunt & Kuffler were invariably studying the same phenomenon as Matthews. The question then arises as to whether or not the slightly supramaximal stimuli used by Matthews excited the gamma motor fibres, for on the basis of more recent work (Leksell, 1945; Kuffler *et al.* 1951) on the relative thresholds of α and γ motor nerve fibres it might be suggested that his stimuli were too weak to have done so. Matthews's results might then perhaps be attributed to the specific activation of the spindle by some group of nerve fibres which were larger than the γ fibres, for the existence of such a motor supply to the spindle has recently been suggested (Granit, Pompeiano & Waltman, 1959*a*, *b*; Boyd, 1959).

Thus the classification of muscle spindle endings into groups A 1 and A 2 has not been satisfactorily explained, and still presents certain interesting features. Matthews's experiments with tetanic stimulation of the muscle nerve have therefore been repeated to see whether his classification can be confirmed, and in order to compare the thresholds of the stimuli required to elicit A 2 responses with the threshold of the γ motor fibres determined under similar experimental conditions. As described in a preliminary communication (Harvey & Matthews, 1960) it has been found that virtually all muscle spindle endings can give an A 2 response and that this can be attributed to the excitation of γ motor fibres, though in some cases 'α-excitation' of the ending similar to that analysed by Hunt & Kuffler was also observed. Some of the observations are also of interest in relation to current problems of the innervation of the muscle spindle.

METHODS

Preparation. The experiments were performed on twelve cats. Eleven were anaesthetized with pentobarbitone sodium (Nembutal; Abbott Laboratories) given intraperitoneally, and one was decerebrated. The discharge of single muscle spindle endings lying in the soleus muscle was recorded under paraffin from thin dorsal root filaments, exposed by lumbar laminectomy and split under a dissecting microscope. Soleus was completely isolated from the spinal cord by cutting the L 6, L 7, S 1, S 2 dorsal and ventral roots, except in the case of the decerebrate cat in which part of one ventral root unfortunately escaped section (as the behaviour of the six endings then studied was typical the results were included in the series). Isolation of single units was aided by widespread denervation of leg and hip muscles. Muscle spindle afferents were identified by the slowing of their discharge occurring during a submaximal twitch of soleus elicited by stimulating its nerve. The conduction distance was determined at the end of the experiment by dissecting out the sciatic nerve with its attached dorsal roots. In calculating the velocity no correction was made for any possible utilization time intervening between the beginning of the 0·1 msec stimulating pulse and the

initiation of the action potential. One possible source of error in this determination was observed for smaller afferent fibres with relative high thresholds to electrical stimulation. These were sometimes excited ephaptically by the muscle action potential (Lloyd, 1942; Granit *et al.* 1959*b*) when the stimulus was too small to excite them directly, and thus appeared to have an unduly long conduction time (cf. Lundberg & Winsbury, 1960). This artifact was readily recognizable, as on progressive increase of the stimulus strength the conduction time suddenly shortened to its true value.

Altogether 105 endings were studied, but they were not all studied under a full range of experimental conditions. The series probably contains too many primary endings in relation to their frequency of occurrence in the muscle, even though particular care was taken to obtain endings with slowly-conducting afferent fibres; but the series undoubtedly contains muscle spindle afferent endings of all kinds as the conduction velocities of the afferent fibres studied were well scattered between 23 and 116 m/sec (see Fig. 3, and Hunt, 1954). Following Hunt (1954), and in order to facilitate description of the results, a conduction velocity of 72 m/sec has been arbitrarily taken as the dividing line between afferents from primary and afferents from secondary endings.

Myography. The leg of the cat was held rigidly by pins in each end of the tibia. The pelvis was steadied by pins in the iliac crests. The tendon of the soleus muscle was dissected free from that of gastrocnemius and was connected by a rigid link to an isometric myograph. The myograph utilized a mechano-electric transducer valve (RCA 5734) the output of which was amplified and displayed on a cathode-ray tube. The soleus was separated from the lower part of gastrocnemius, which was denervated, and the skin was sewn back over the muscles, leaving the tendon protruding. The temperatures of the muscle, of the paraffin pools and of the cat were usually in the range 36–38° C.

Stimulation. The nerve to soleus was dissected out of the lateral head of gastrocnemius through which it runs and was covered by liquid paraffin contained by flaps of skin and muscle. The stimulating electrodes were applied to the nerve 1–2 cm away from its point of entry into the soleus, and with the cathode closer to the muscle than the anode. The electrodes consisted of a pair of silver wires separated by about 5 mm. The stimuli were square pulses of 0·1 msec duration and were isolated from earth by being delivered through a transformer. The intensity of the stimuli was controlled and determined by means of a calibrated potentiometer. Probably because of the presence of stray connective tissue it was sometimes found that small movements of the nerve on the electrodes produced appreciable changes in the effectiveness of the stimulus. To circumvent this difficulty frequent determinations were made of the threshold of the α motor fibres to single stimuli (by observing the contraction of the muscle), so that comparisons of the strength of stimuli delivered at different times could be made in terms of their relation to the α threshold rather than in absolute units. The absolute value of the threshold of the α motor fibres was always appropriately low and varied from 35 to 160 mV in different experiments. The strength of stimulus required to produce a maximal contraction of the muscle was also frequently checked.

Recording. The action potential of the afferent fibre studied was amplified by conventional means and, in order to facilitate its observation during a tetanic contraction, was displayed on two separate cathode-ray tubes. One tube had a sweep lasting 2–3 sec and the tension developed by the muscle was displayed on its second beam. This record gave a general picture of the time relations between the discharge of the ending and the contraction of the muscle. The second tube had a sweep lasting about 50 msec and allowed the time relations between the stimulating pulses and the action potentials to be determined. This tube received triggering pulses from the stimulator, arranged so that during and only during the period that the nerve was being stimulated the beam was sweeping repetitively with the stimulus artifacts occurring at constant points on the tube face. During a 0·8 sec period of tetanic stimulation 10–15 of these repetitive traces were superimposed in a single photo-

graphic record, alongside a record of the cathode-ray tube with the slow sweep. This method of display was used largely because it was economical of recording paper. When necessary the cathode-ray tube sweeps were stopped and records were taken on photographic paper moving at 18 cm/sec, using about ten times more recording paper. During observations on any one ending a 0·8 sec train of stimulating pulses was delivered every 10 sec. Between consecutive records either the intensity or the frequency of the stimulus could be altered while the camera moved on. This pattern of stimulation was continued for periods of 1–2 min without any significant signs of fatigue of the muscle or of the ending. The natural stimulus artifact was usually very small and so an artificial stimulus artifact was frequently introduced into the action potential records on one or both cathode-ray tubes. Most endings were studied both with the muscle just taut (tension under 50 g) and with the muscle extended to within a few mm of its physiological full extension (tension up to 500 g), except for some secondary endings with high thresholds for tension, which were only studied under appreciable tension. Stimulus frequencies of 1, 30, 50, 70, 100/sec were regularly used at three different intensities of the stimulus. The intensities used were first, sufficient to produce a half to three-quarters maximal contraction of soleus; secondly, about 120–140 % of that required to produce a maximal contraction; and thirdly, about ten times that required to produce a threshold contraction of the muscle (about seven times maximal). Lower frequencies of stimulation and intermediate values of stimulus intensity were also employed on many occasions.

RESULTS

Response to tetanic stimulation

In all but two of the ninety muscle spindle endings studied in this respect tetanic stimulation of the muscle nerve with shocks strong enough to excite the γ motor fibres elicited an A 2 type of response, provided that the initial tension and the frequency of stimulation were appropriately adjusted. The afferent fibres studied had conduction velocities ranging from 23 to 116 m/sec, and it may therefore be concluded that the A 2 response is given both by the primary and by the secondary endings of the muscle spindle (Hunt, 1954). Figure 1 shows a typical A 2 response from a primary ending. The top traces (*a*) show the tension developed in soleus on stimulating its nerve with shocks slightly supramaximal for eliciting a contraction. Immediately beneath the myographic traces, and recorded on the same sweep speed, lie the records of the responses of the single muscle spindle afferent fibre, recorded from a dorsal root filament. During the twitch contraction the discharge of the muscle spindle ending ceased. During the tetanic contraction, though nerve impulses were still recorded, they were not set up at the ending but were due solely to the direct electrical stimulation of the afferent fibre studied. This is shown by the superimposed traces *c*, which record the discharge of the afferent fibre during the period of stimulation with a high-velocity sweep locked to the stimulus. It can be seen in this record that all the spikes follow the stimulus artifacts with a short constant latency. Traces *d*, *e*, and *f* were recorded similarly to *a*, *b*, and *c*, but were taken when the stimulus strength was considerably supramaximal. In this case, during the tetanic contrac-

tions, there are numerous impulses in addition to those set up by the direct electrical stimulation of the afferent fibre, and these must have been initiated at the nerve ending within the muscle spindle. Figure 2 shows a similar response from a secondary ending on increasing the strength of the stimulus.

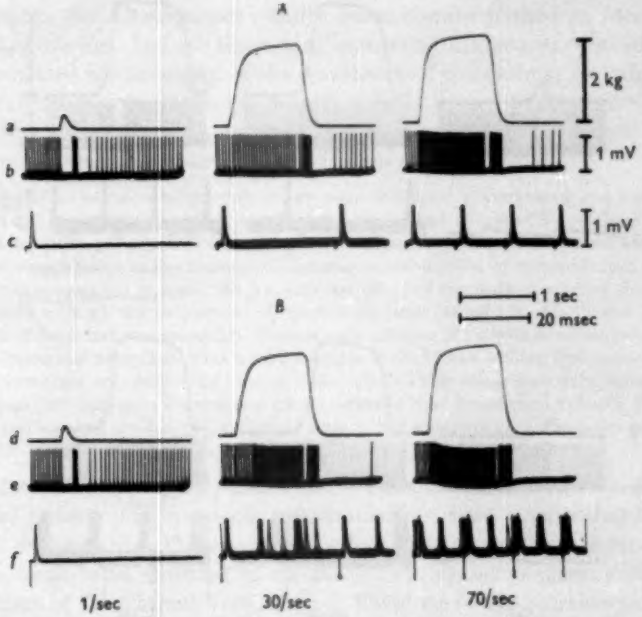

Fig. 1. An A 2 response given by a primary ending (afferent fibre conduction velocity 103 m/sec). *A*, absence of A 2 response on slightly supra-maximal stimulation of muscle nerve (stimulus 1·5 times α threshold). *B*, presence of A 2 response on greatly supramaximal stimulation (stimulus 10 times α threshold). *a*, *d*, myographic records of tension in soleus; *b*, *e*, action potentials of single afferent fibre; these four traces are all on the same slow sweep speed (see upper time scale). *c*, *f*, superimposed traces showing the action potentials of the single afferent fibre occurring during the period of stimulation, but recorded at a high sweep speed (see lower time scale) and with the sweep locked to the stimulus. Stimulus artifacts were introduced artificially into the high-speed records, where they form the downward deflexion; the upward deflexions are action potentials in all records. (Initial tension approximately 10 g. Records retouched.)

Such results were typical and were found for every one of the 55 primary endings and for all but two of the 35 secondary endings studied with tetanic stimulation. The only acceptable interpretation of such a result is that the stronger stimuli excited fusimotor nerve fibres, with a consequent stimulation of the afferent endings of the muscle spindle. The findings thus

confirm previous work showing, by means of stimulation of single γ motor fibres, that both the primary and the secondary endings of the muscle spindle may be so excited (Kuffler *et al.* 1951; Hunt, 1954).

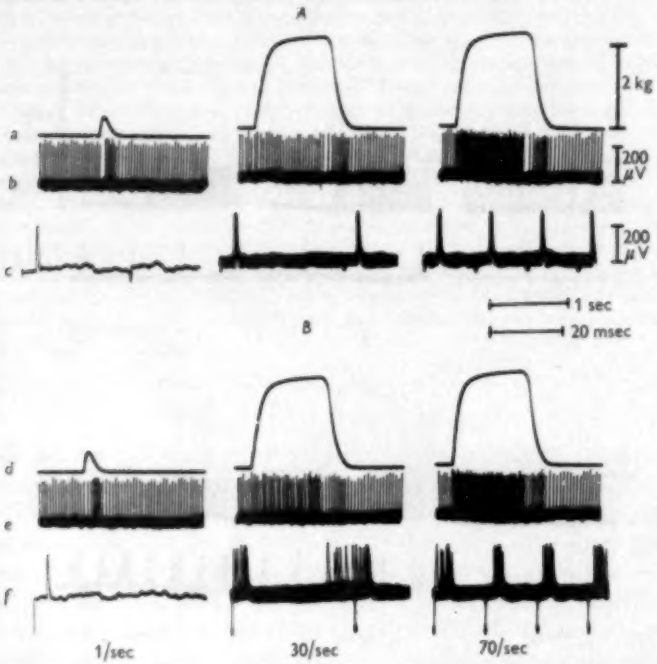

Fig. 2. An A 2 response given by a secondary ending (afferent fibre conduction velocity 45 m/sec). *A*, absence of A 2 response on moderately supramaximal stimulation of muscle nerve (stimulus 2·5 times α threshold). *B*, presence of A 2 response on greatly supra-maximal stimulation (stimulus 12·5 times α threshold). *a*, *d*, myographic records of tension; *b*, *e*, action potentials recorded on same slow sweep speed as myographic records; *c*, *f*, superimposed traces on a high sweep speed of the action potentials occurring during the period of stimulation. Stimulus artifacts present only in the high speed records, where they form the downward deflexions. Initial tension 150 g. (Records retouched.)

In many cases, however, it proved more difficult to produce the A 2 response from secondary than from primary endings. Also the A 2 response of the secondary endings was in general rather weaker than that of the primary endings. For example, in Figs. 1 and 2 it can be seen that the time interval between the direct spike elicited by the stimulus and the subsequent spike initiated at the ending was appreciably longer for the secondary ending, and such a difference was common. In addition,

whereas primary endings usually gave an A 2 response at initial tensions of under 50 g, secondary usually only gave an A 2 response if the initial tension on the muscle was 50–300 g, and in six cases tensions of 300–900 g were required. (For example, the ending of Fig. 2 did not show an A 2 response when the initial tension was 30 g.) For both primary and secondary endings the A 2 responses usually became more marked on increasing the initial tension. In both cases, also, increasing the frequency of stimulation increased the intensity of the excitation of the ending, as judged by a decrease in the time interval from the direct spikes to those evoked at the ending (Figs. 1 and 2); and in the case of a few secondary endings an A 2 response was only found on stimulation at 50/sec or above.

On stimulation at frequencies below 20/sec weak excitation of the ending was sometimes observed, but its detection was hindered by the mechanical effects of the unfused tetanic contraction of the extrafusal fibres. Frequencies of stimulation above 100/sec were only employed occasionally, as the consequent increase in the number of spikes elicited directly by the stimulus tended to mask the A 2 response. Most of the endings studied showed an A 2 response with all the frequencies of stimulation used (usually 30, 50, 70 and 100/sec, often also 20/sec), but two secondary endings only gave an A 2 response on stimulation at 70 and 100/sec and two others gave an A 2 response at 50, 70 and 100/sec, but not at 30/sec. In these four cases only one initial tension was studied. Three other secondary endings and two endings just falling in the primary group (afferent fibre conduction velocity 73 m/sec in each case) behaved similarly at one initial tension, but also gave an A 2 response at 30/sec stimulation when the tension was increased.

The fact that the A 2 response was not detected under some conditions of initial tension and frequency of stimulation, even when using strong stimuli, suggests that the excitatory effect of the intrafusal contraction was too weak to be detected in the face of simultaneous direct electrical stimulation of the afferent fibre studied. Each one of the impulses thereby excited would travel antidromically to the ending and reset its rhythm (Matthews, 1933) so that the discharge of the ending could only be detected when it was of higher frequency than that of the electrical stimulus (except for a few cases of secondary endings with axons conducting at about 30 m/sec whose A 2 threshold was slightly below that of the afferent fibre). In addition, before producing any excitatory action the intrafusal contraction must cause sufficient shortening to overcome the unloading of the spindle due to the contraction of the extrafusal muscle fibres.

Thus the finding of two secondary endings which did not show the A 2 response under any of the conditions studied cannot be taken to mean that no intrafusal effect was present. Indeed, the occurrence of some weak γ excitation was strongly suggested in one of these cases by the finding that on increasing the stimulus strength to considerably above α maximum the ending fired more rapidly at the beginning of the relaxation of the tetanic contraction than it did with weaker stimuli (cf. Matthews, 1933). Other endings with axons of similar conduction velocity (34 and 42 m/sec) did show an A 2 response.

Values of the threshold strength of the stimuli required to produce the A 2 response

On progressive increase of the strength of the tetanic stimulus to the muscle nerve the A 2 response appeared at a definite and repeatable threshold. This presumably corresponded to that of some particular motor nerve fibre supplying the intrafusal muscle fibres of the ending studied. The value of the thresholds of the motor fibres exciting different muscle

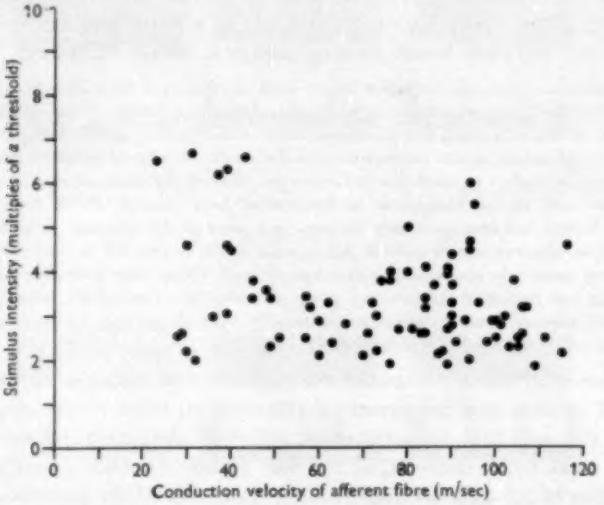

Fig. 3. The distribution of the A 2 thresholds of 84 endings. Abscissa, conduction velocity of the afferent fibre of the ending; ordinate, threshold strength of stimulus required to elicit an A 2 response for the ending. The stimulus strength is expressed in terms of the threshold of the most excitable α motor fibres of the same preparation. A stimulus of 1·6 times α threshold sufficed in all cases to produce a maximal contraction of the muscle.

spindle endings is of interest in relation to current suggestions, based on histological observations, that there are different kinds of intrafusal muscle fibres, with different relations to the primary and the secondary afferent endings, and with motor endings of different types (Barker & Ip, 1960; Boyd, 1959; Cooper, 1960; Cooper & Daniel, 1956). The threshold was expressed in terms of the threshold of the α motor fibres determined at the same time (by observation of the muscle contraction) so that the results of different experiments could be compared. The determination was made with a frequency of stimulation of 70/sec (rarely 50 or 100/sec), as at this frequency the occasional weak excitatory effect of stimulating

α fibres (see later) was masked by the impulses directly excited by the electrical stimulus, while this frequency also favoured the γ excitation of the ending. The initial tension was not closely controlled for this determination; for primary endings it was usually 50–150 g, while for secondary endings rather higher tensions were sometimes necessary (*vide supra*). The distribution of the A 2 thresholds of 84 endings (54 primary, 30 secondary) is shown in Fig. 3, where the strength of the stimulus required to produce an A 2 response is plotted against the conduction velocity of the afferent fibre concerned. A maximal contraction of the muscle was produced by stimuli varying in different preparations from 1·2 to 1·55 times α threshold (maximal response was gauged by size of twitch). The lowest threshold found for an A 2 response was 1·7 times α threshold, and there were only four values below 2·0, so that all the A 2 thresholds plotted can be attributed to excitation of motor fibres with a higher threshold to electrical stimulation than the α fibres. At the end of eight of the twelve experiments the relative thresholds of the different sized motor fibres were sought under existing experimental conditions by recording monophasically their compound action potential from the peripheral end of an appropriate cut ventral root (L 7 or S 1) on stimulating the nerve to soleus. The stimulating electrodes remained undisturbed in the position used when studying the single afferent fibres, but the nerve was crushed at its point of entry into the muscle in order to eliminate the 'back-response' (Leksell, 1945; Brown & Matthews, 1960). The threshold of the least detectable γ wave was 2·1–2·4 times α threshold in five experiments, 1·8 and 1·6 times α threshold in two other experiments; in one experiment the γ wave was so small that it could not be detected with certainty and its threshold could not be determined. These low-threshold γ fibres had conduction velocities, calculated from the shock-response interval and the conduction distance, of 40–45 m/sec. It may be concluded that all the A 2 responses can be attributed to excitation of γ motor fibres.

The γ wave was identified by its occurrence after the α wave (though it sometimes fell in the after-potential of the α wave), and by its requiring a stronger stimulus than that required to produce an α wave of maximum amplitude (Leksell, 1945). The detection of the threshold of the γ wave was facilitated by recording it at five to ten times the amplification used for the simultaneously recorded α wave, and also by superimposing the traces of several sweeps photographically. The determination of the γ threshold was usually made with a stimulus frequency of 2 or 5/sec, but no difference in threshold was found on the one occasion on which the frequency was increased to 70/sec. The form of the α wave was not carefully studied, and the sweep speed used was not high enough to enable small changes in its falling phase to be detected on increasing the stimulus strength.

Crushing the nerve to eliminate the 'back-response' sometimes produced a temporary lowering of the threshold of the α fibres, presumably because of the flow of injury current, but observations on threshold were usually made when the threshold had returned to roughly its previous value. A single observation suggested that slight changes might occur

in the relative thresholds of α and γ fibres as a result of crushing, but because of the difficulty of studying the γ wave in the presence of the back-response no systematic observations were made of its threshold in the intact nerve. In another experiment the effect of the proximity of the stimulating electrode to the crushed end was investigated by determining the relative thresholds of the α and γ fibres, both before and after moving the electrodes 0·5 cm. This procedure made little difference to the relative thresholds and a similar result was obtained when several positions of the stimulating electrode were studied in an experiment on the nerve to the medial head of the gastrocnemius. It is felt, therefore, that the effect of

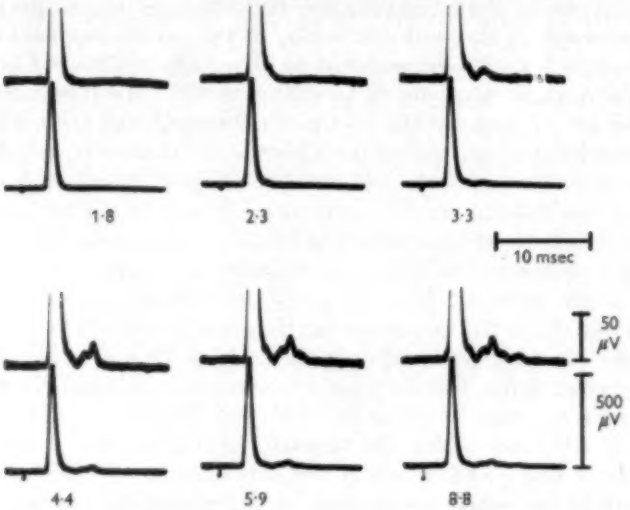

Fig. 4. Records showing the progressive prolongation of the γ wave on increasing the stimulus strength. The compound action potential of the soleus motor fibres was recorded monophasically from the L 7 ventral root on stimulating the soleus nerve. Records were taken simultaneously at two different amplifications in order to favour the display of either the γ wave (above) or the α wave (below). The stimulus strength, in terms of α threshold, is given below each pair of records. (Each record consists of about 15 superimposed traces. Stimulus artifacts introduced into low-gain traces, but not into high-gain traces. Records retouched.)

crushing is unlikely to have invalidated the results, but that no great significance should be attached to the precise value of the γ threshold relative to the α threshold or to the difference of the γ threshold found in the different experiments.

The values, relative to α threshold, of the stimuli required to give a maximal α wave or a threshold γ wave are both rather lower than those found by Leksell (1945). This difference is probably partly due to the fact that Leksell did not study the soleus nerve. This nerve differs from many other motor nerves in that the α motor fibres are smaller than usual, while the γ motor fibres are not (Eccles & Sherrington, 1930). Such an explanation is favoured by our finding in five experiments on the nerve to the medial head of gastrocnemius that the values of γ threshold and of α maximum tended to be higher relative to the α threshold than in soleus nerve (γ threshold 1·8, 2·5, 2·6, 2·8, and 3·3 times α threshold; α maximum 1·2, 1·8, 1·8, 1·8 and 2·0 times α threshold); but the values obtained were lower than that found

by Leksell for a single experiment on this nerve (γ threshold 4·0; α maximum 3·3). There were probably, however, differences in the stimulating arrangements used in the two sets of experiments.

Figure 3 shows that the A 2 thresholds of different endings differed considerably, but that there was no sharp discontinuity between the distribution of the A 2 thresholds of the primary and secondary endings. In both cases many endings gave an A 2 response with stimuli at or below three times α threshold, showing that they were excited by low-threshold

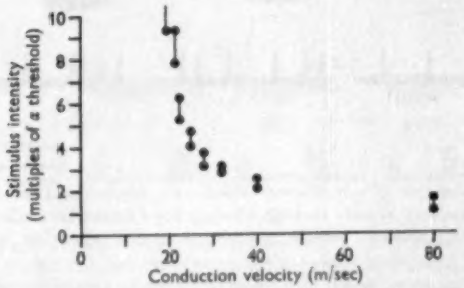

Fig. 5. The strengths of stimuli needed to excite γ motor fibres of different conduction velocities in the experiment of Fig. 4. Abscissa, conduction velocity of various parts of the γ wave; ordinate, strength of stimulus, in terms of α threshold, required to excite different parts of the γ wave. The two points at each conduction velocity correspond to the stimulus required to excite the least detectable γ wave and that required to excite a maximal γ wave of that velocity. (The records used for measurement were taken at a higher sweep speed than those illustrated in Fig. 4.)

γ fibres. In both cases also a few endings had A 2 thresholds above five times α threshold, showing that they were excited by high-threshold γ fibres. Leksell (1945) showed that the more slowly conducting γ fibres had a higher threshold to electrical stimulation than did the more rapidly conducting γ fibres. This was confirmed for the present conditions of stimulation and Figs. 4 and 5 show the relation between the electrical threshold and conduction velocity of various parts of the γ wave found in one experiment. Six other experiments gave similar results, though in only one of them was this wide range of stimulus strengths employed, either because in these cases the strong stimuli 'spread' so as to excite the cut nerve fibres from the lateral head of gastrocnemius, or because the tail of the γ wave was not clearly detectable. In order to discuss the significance of the A 2 thresholds the results expressed in Fig. 5 have been taken to apply approximately to all the experiments. Thus stimuli of three times α threshold excited γ fibres with conduction velocities of 30–48 m/sec, corresponding to fibre diameters of 5–8 μ (Hursh, 1939).

Stimuli above five times α threshold excited γ fibres with conduction velocities below 25 m/sec, corresponding to fibre diameters of less than 4 μ.

It is known, of course, that a single ending can usually be excited by several different γ fibres (Hunt & Kuffler, 1951*a*; Whitteridge, 1959).

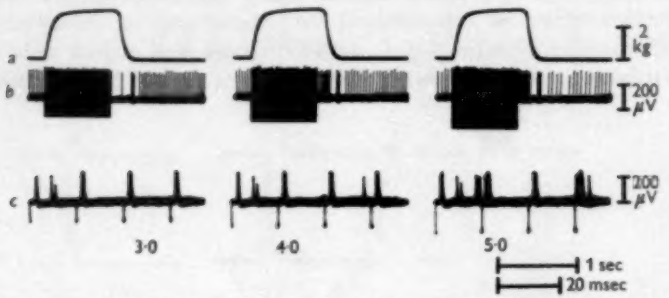

Fig. 6. An example of γ excitation which was subthreshold for the production of an A 2 response (primary ending, afferent fibre conduction velocity 94 m/sec). *a*, myographic records of tension in soleus; *b*, action potentials of afferent fibre recorded on same slow sweep speed as myographic records; *c*, superimposed traces on a high sweep speed of the action potentials occurring during the period of stimulation. Frequency of stimulation 70/sec in all cases. Intensity of stimulation, in terms of α threshold, marked below each set of records, and appreciably supramaximal for the contraction in all cases. The weakest stimuli (left) directly excited the afferent fibre of the ending, and after the last spike so excited only one impulse occurred on the falling phase of the contraction. In addition, the first stimulus of the train excited an 'early discharge' of two impulses, the second being smaller than the first because it followed it so closely, and this was also seen with the stronger stimuli. The intermediate strength stimuli (centre) were followed by a burst of impulses on the falling phase of the contraction, thereby demonstrating γ excitation of the ending; but there was no A 2 response during the period of stimulation. The strongest stimuli (right) caused a typical A 2 response followed by a greater burst of impulses on the falling phase of the contraction. (Stimulus artifacts downward and present in all records. Initial tension, 70 g. Records retouched.)

The γ fibre whose recruitment on increasing the stimulus just suffices to produce an A 2 response from a particular ending need not be the lowest-threshold γ fibre affecting that ending, for weak excitatory effects on an ending might pass undetected, as already discussed. In a few cases it was clearly apparent, on subsequent inspection of the records, that the ending had indeed been excited by a γ fibre of lower threshold than that whose excitation produced the overt A 2 response. For increasing the strength of stimulus already supramaximal for the α fibres caused the ending to fire for a short time after the last of a train of stimuli, while there was no such discharge with weaker stimuli. A striking example is illustrated in Fig. 6. (Such effects were usually less prominent, as most endings

discharged on the falling phase of the contraction with all strengths of stimulation.) In addition, in a few experiments when the initial tension on the muscle was increased the A 2 threshold of the ending fell to a new, lower value, possibly because the excitatory effect of a lower-threshold γ fibre was then sufficiently facilitated by the stretch to cause an A 2 response without the assistance of the higher-threshold γ fibre. Thus it is probable that many of the endings which gave an A 2 response only on stimulation of small γ fibres were also excited by larger γ fibres.

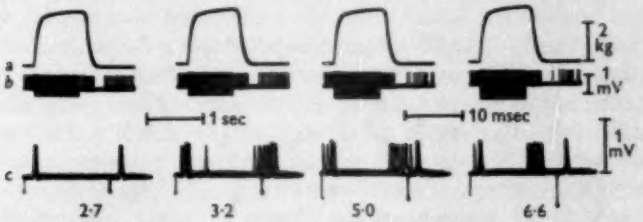

Fig. 7. An example of an increasing excitatory effect on an ending on increasing the strength of the stimulus above the A 2 threshold. (Primary ending, afferent fibre conduction velocity 100 m/sec.) *a*, myographic records; *b*, action potentials at same slow sweep speed as myographic records; *c*, superimposed traces on a high sweep speed of the action potentials occurring during the period of stimulation. Frequency of stimulation 70/sec in all cases. All stimuli were appreciably supramaximal for contraction; their strength, in terms of α threshold, is shown below each set of records. The weakest stimuli (left) were below the A 2 threshold and only excited spikes by direct stimulation of the afferent fibre. All the stronger stimuli excited an A 2 response, but increasing the strength of the stimuli shortened the interval at which impulses initiated at the ending followed the direct spikes. (Stimulus artifacts downward in all records. Initial tension, 45 g. Records retouched.)

It was also observed that many of the endings which gave an A 2 response on stimulation of large γ fibres were further excited by smaller γ fibres, for on increasing the stimulus strength beyond the A 2 threshold a more marked A 2 response was produced, and the latency between the direct spikes and those initiated at the ending decreased. An example of this is shown in Fig. 7, where in each case the increased effect occurred at a definite threshold strength of stimulus and so may be attributed to the recruitment of an additional γ fibre. For one ending the cumulative excitatory effects of four different γ fibres were so distinguished and four different 'incremental thresholds' determined. In no case was the A 2 response found to become less marked on increasing the stimulus strength.

For a number of endings care was taken to determine the value of one of these 'incremental thresholds', because it seemed important to demon-

strate unequivocally that primary endings are regularly influenced by smaller γ fibres. (Only 3 of the 54 primary endings in Fig. 3 have A 2 thresholds above 5 times α threshold, and these were all studied in a single experiment.) The results are shown in Fig. 8, where again the A 2 thresholds of the 84 units of Fig. 3 have been plotted, but when an 'incremental threshold' of 4·0 or over was observed (29 cases) its value has been plotted in place of the threshold intensity for the A 2 response. From this graph

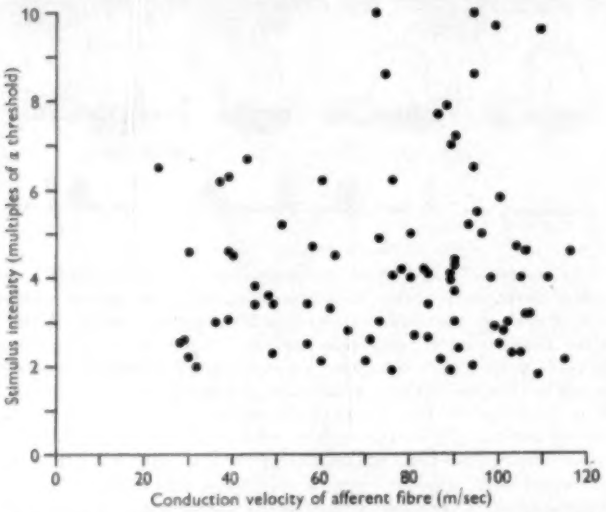

Fig. 8. The distribution for 84 endings of the threshold strengths of the stimuli producing either an A 2 response, or a definite increase in the magnitude of the A 2 response (29 cases). Only one point has been plotted for each ending, and that was the highest value obtained. Abscissa, conduction velocity of the afferent fibre of the ending; ordinate, stimulus strength in terms of the α threshold. (The two values plotted as 10·0 times α threshold were actually slightly higher. Fig. 3 shows all the A 2 thresholds of these 84 endings.)

it may be concluded that both primary and secondary endings may be excited by γ fibres of a wide range of thresholds corresponding to diameters from 4 to 8 μ, or practically the whole of the γ range. (Incremental thresholds of over 10 times α threshold were not sought, partly because, with the present recording arrangement, we were not able reliably to detect small increases in the degree of spindle excitation when they were superimposed on an already large excitatory effect.) Thus the present experiments provide evidence against any suggestion that the γ fibres can be divided into two groups differing both in size and in their action on primary and secondary endings, and the question of the innervation of the

histologically distinct types of intrafusal fibres remains for more direct experimental study.

Response to supramaximal stimulation with single shocks

In the first seven of the twelve cats studied rather little attention was paid to the behaviour of the endings during twitch contractions, though the twitch contractions were regularly recorded on a slow sweep speed, as illustrated in Figs. 1 and 2. Such records showed that the discharge of most endings ceased on the rising phase of the twitch, even though a marked A 2 response occurred at the same initial tension and the same strength of stimulus on repetitive stimulation (Fig. 1). (During the falling phase of the twitch some endings fired a burst of impulses with all strengths of stimuli, other endings were silent.) Such behaviour on the rising phase was described by Matthews (1933) and is not surprising in view of the slow rate of contraction of intrafusal muscle fibres and of their relative unresponsiveness to single-shock stimulation of γ motor fibres (Kuffler *et al.* 1951; Eyzaguirre, 1960). Matthews also sometimes found that on increasing the initial tension supramaximal stimulation caused the silence of the A 2 endings on the rising phase of the twitch to be replaced by an acceleration of the discharge. Such an acceleration may, however, apparently be produced by stimulating α fibres alone (Hunt & Kuffler, 1951*b*; Hunt, 1954; also observed in the present experiments); and in addition α fibre stimulation may excite an 'early discharge' of the spindle afferent at the foot of the contraction (Hunt & Kuffler, 1951*b*; Granit *et al.* 1959*b*). In view of these complications, and as Matthews's classification had been based primarily on the behaviour of the ending during tetanic stimulation, we did not initially feel that it was profitable to attempt to make endings fire on the rising phase of the twitch, and thereby to confirm the results obtained with tetanic stimulation. Very recently, however, Diete-Spiff (1960) in preliminary experiments on the rabbit found that while, with the aid of partial neuromuscular block with succinylcholine, all primary endings could be made to fire on the rising phase of a twitch, secondary endings could never be made to do so, though after giving succinylcholine they could be excited by tetanic stimulation to give responses of an A 2 type. He related this to the histological work of Boyd (1959) suggesting that secondary endings lie almost exclusively on small intrafusal muscle fibres which were presumed to give only non-propagated contractions. In the last five experiments we therefore studied more carefully the behaviour of both secondary and primary endings during the twitch.

Secondary endings. By using strong stimuli and an appreciable initial tension we found that 11 out of 24 secondary endings studied in this manner

could be excited to fire one or two impulses on the rising phase of the twitch. A typical example of such excitation is shown in Fig. 9. The effect has a threshold in the γ range and can be attributed to excitation of γ motor fibres; it cannot be attributed to repetitive firing of the afferent fibre induced by the electrical stimulus for the duration of this was 0·1 msec. The threshold was not regularly determined, but it tended to be higher than the A 2 threshold determined with repetitive stimulation, though it was not always as high as that for the ending of Fig. 9. The demonstration of excitation of the ending on the rising phase of the twitch was favoured by increasing the initial tension. Six secondary endings were excited at initial tensions of less than 300 g, while five other endings

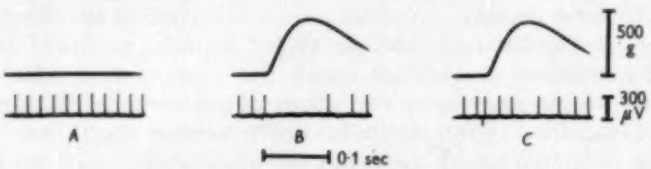

Fig. 9. The response during twitch contractions of a secondary ending (afferent fibre conduction velocity 53 m/sec) which discharged on the rising phase of the contraction when the stimulus was strong. Above, myographic records; below, action potentials recorded on the same sweep speed. *A*, resting discharge at the initial tension of 350 g; *B*, stimulus 4 times α threshold; *C*, stimulus 13 times α threshold. (Records retouched.)

required initial tensions ranging from 350 to 830 g. The remaining 13 secondary endings studied did not show the effect even with strong stimuli when tested with initial tensions of between 300 and 800 g. The effect was best shown when the stimulus was regularly repeated at a rate of 1 or 2/sec, and was often absent from the first of a train of twitches. The conduction velocity of the afferent fibres of the secondary endings which fired on the rising phase ranged from 23 to 65 m/sec, while for those which were silent it ranged from 34 to 66 m/sec.

Primary endings. In contrast with the above results not one of 18 primary endings studied under similar conditions produced any γ-evoked impulses on the rising phase of the twitch with stimuli of 14 times α threshold and initial tensions of about 300 g, approximately corresponding to full physiological extension. (Some had 'early discharges' occurring at the foot of the contraction, and one fired a single spike on the rising phase, but both these effects were produced by stimuli too weak to have excited γ fibres.) For 15 of these endings it was confirmed that they gave normal A 2 responses on repetitive stimulation; moreover it was apparent for 9 of them that the single shock stimulation of the γ fibres had produced

a slight excitatory effect, for the first impulse to be fired on the falling phase of the twitch occurred just perceptibly earlier (about 5 msec) when the stimulus was greatly supramaximal. A number of these endings were tested at initial tensions of up to 500 g without firing on the rising phase. When two were tested at an initial tension of 1 kg one ending fired on the rising phase but the other did not. No doubt more primary endings could have been induced to fire on the rising phase of the twitch by using such large tensions, but this seemed of little value and might have damaged the muscle. (A tension of 1 kg approximately halved the twitch tension, and stretched the muscle well beyond its physiological range.) The interesting finding is that the primary endings of soleus are, in comparison with the secondary endings, extremely reluctant to fire on the rising phase of the twitch; while with tetanic stimulation it is generally easier to demonstrate the excitatory effect of γ fibre stimulation upon primary than upon secondary endings.

Additional results relating to the possible existence of 'α-innervation' of muscle spindles

The question whether or not some muscle spindles are innervated by motor nerve fibres of α diameter is at present controversial. While it is generally agreed that stimulation of α fibres without γ fibres may excite some spindles, the significance of this observation is in doubt. Hunt & Kuffler (1951*b*) and Hunt (1954) distinguished two types of 'α-excitation' of both primary and secondary endings. The first consisted of very varied patterns of discharge of the ending during the rising phase of twitch or brief tetanic contractions. Such discharges were found only when the muscle was under high initial tension, and were attributed to 'tension changes on the spindle produced by the contraction of extrafusal muscle fibres'. Secondly, they described the 'early discharge' occurring at the foot of the contraction and attributed it to the stimulating effect on some spindles of 'early tension changes within the muscle', and not to any specific innervation to the spindles. More recently Granit *et al.* (1959*b*) suggested that the early discharge might consist of two separate components, an initial spike of short latency attributable to ephaptic excitation of the ending (or its afferent fibre) by the muscle action potential, and later spikes attributable to stimulation of the ending by a specific α motor supply to the spindle. In the present experiments both types of 'α-excitation' have been periodically observed for both primary and secondary endings on stimulating the nerve with shocks too weak to have excited γ motor fibres. We do not feel that experiments such as ours can resolve the problem of their interpretation, but certain findings are of interest in this respect.

Early discharges. The 'early discharge' was seen in slightly under half the endings studied. It consisted of 1–4 impulses, was maximally developed with stimuli just maximal for α fibres, and did not increase further on increasing the stimulus so as to excite γ motor fibres. Secondary endings with axons conducting at below 45 m/sec only occasionally had an 'early discharge', and this never consisted of more than one impulse. Otherwise an 'early discharge' was about equally common for primary and for secondary endings. The 'early discharge' was much better shown on stimulation with single shocks, than on repetitive stimulation. On stimulation at 30/sec only two cases were observed in which more than the

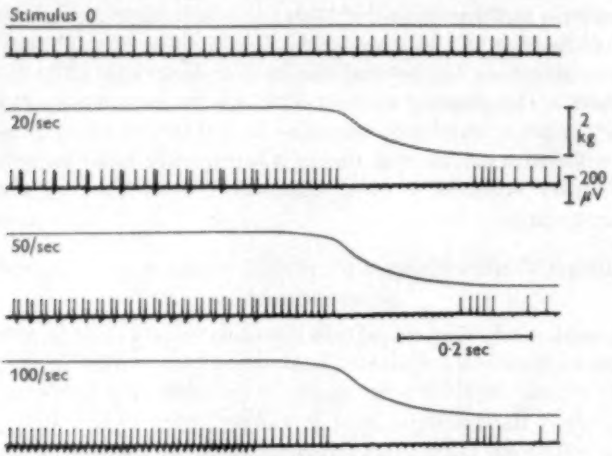

Fig. 10. Four pairs of records showing the typical slight effect, on the intensity of 'α-excitation' of an ending, of altering the frequency of stimulation (primary ending, afferent fibre conduction velocity 98 m/sec). The records show the resting discharge (top), and the last part of tetanic contractions elicited by three different frequencies of stimulation. In each case the myograph record is above, and the action potential record below; they were recorded simultaneously on moving paper. The stimulus intensity was in all cases 1·6 times α threshold, which was slightly supramaximal for contraction but below the threshold of any γ fibres. (In the action potential records the large downward deflexions are the stimulus artifacts; the small downward deflexions are spikes from another ending. Initial tension, 190 g. Records retouched.)

first spike of the early discharge persisted after the first few stimuli (cf. Granit *et al.* 1959*b*), and often the first spike did not persist either (for example see Fig. 6). Any spike of the early discharge which persisted on repetitive stimulation occurred with an almost constant latency just after the spike directly set up by the stimulus. In this it differed from the spikes evoked by γ fibre stimulation, for these appeared rather later with a variable latency, and the two responses could therefore be clearly distinguished. The first spike of the early discharge is probably due to ephaptic stimulation by the muscle action potential (Granit *et al.* 1959*b*), as certainly occurs in the case of motor fibres (Lloyd, 1942; Leksell, 1945; Brown & Matthews, 1960). The failure of the later spikes of the early discharge to persist during tetanic stimulation shows that if these should be due to a true α innervation of the spindle, and not to 'early tension changes' or further ephaptic stimulation, then the excitatory effect of this α innervation is weak in comparison to that of γ innervation.

Occasional 'α-excitation' on low-frequency stimulation. Another pattern of 'α-excitation', prominent only on low-frequency stimulation, was seen in 12 endings (8 primary, 4 secondary) of the 75 which were studied with the full range of stimulus frequencies (most of these also had an early discharge, though this was not maintained on stimulation of 30/sec). This is illustrated in Fig. 10, which shows the response of a primary ending at the end of tetanic contractions elicited by stimuli slightly above maximal for the contraction, but below the γ threshold. The ending fired at a slightly higher rate during the stimulation than at the initial length, and this discharge continued for approximately 100 msec after the last stimulus while the tension in the muscle was still maintained. The interesting and typical feature of this response is that it was barely increased in rate by increasing the frequency of stimulation. This is most clearly shown by comparing the frequencies of discharge in the periods after the last stimuli, but is also shown during the period of stimulation. Comparable insensitivity to increasing the frequency of stimulation above 20/sec was found in all but one of the 12 cases of α excitation and contrasts with the marked effect of increasing the frequency of stimulation of γ efferents. It suggests that the α excitation of the ending results from the contraction of muscle fibres with a low tetanic fusion frequency, in the same range as that of the extrafusal muscle fibres of soleus.

In one exceptional case, that of an ending with an afferent fibre conducting at 49 m/sec, an effect typical of γ excitation, favoured by increasing the frequency of stimulation, was produced by a submaximal stimulus 1·3 times α threshold. The α excitation of this ending was so great that superadded γ excitation could not be detected (it has been included in the series as giving an A 2 response, though its A 2 threshold was not included in Figs. 3 and 8). If such behaviour were at all usual it would have been more frequently observed in the present experiments, for 90 endings were tested with tetanic stimulation just above α maximum (15 were only tested at 70/sec, but the rest were tested with a range of frequencies). We can only suppose that in this case a nerve fibre with an ending on the intrafusal muscle fibres was of α diameter; alternatively it seems possible that a γ efferent fibre had a low threshold for some unknown reason. We do not feel that this exceptional case provides evidence for the existence of α innervation of the spindle on a physiologically significant scale.

Otherwise, the intensity of α excitation of any ending was weaker than γ excitation of the same ending, and was usually weaker than that shown in Fig. 10; in addition, α excitation did not usually produce a discharge continuing so long after the last stimulus as that in Fig. 10. On stimulating at 70/sec it was usually masked by the directly excited spikes, and so did not then interfere with the detection of γ excitation of the same ending. The threshold of the stimulus required to elicit α excitation varied for different endings and was not always sharp, possibly because submaximal stimulation did not always give a steady tetanic contraction and this confused the picture. Precise determination of the threshold was not usually attempted, but it was noted usually to be well below α maximum, and sometimes close to α threshold. In a few cases α excitation was produced by submaximal but not by maximal stimuli. Some other endings, on stimulation of α fibres alone, fired a few impulses on the rising phase of a twitch or of a tetanic contraction, but this discharge did not persist during a maintained tetanic contraction. All these α effects were very dependent upon the initial tension, and were often absent at low tensions.

The certain absence of 'α-excitation' of some secondary endings. It might be suggested that weak α excitation of the endings occurred invariably but was concealed by the antidromic impulses set up by the direct electrical stimulation of the afferent fibres of the endings. For the primary endings such a suggestion cannot be excluded, since their afferent fibres had low thresholds to electrical stimulation, and a maximal muscle contraction could not be produced without exciting them. The threshold to electrical stimulation of the afferent fibres of many secondary endings was, however, rather higher, and it was usually possible to produce a maximal contraction without exciting those conducting at less than 60 m/sec. In this case an excitatory α innervation should be detectable, for if of physiological

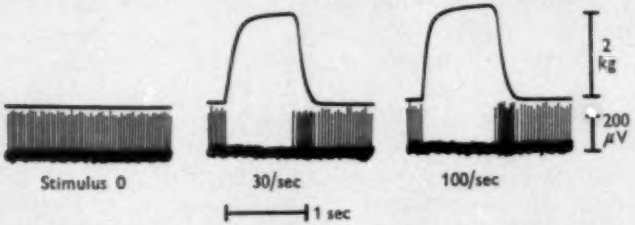

Fig. 11. The response of a secondary ending (afferent fibre conduction velocity 45 m/sec) showing no sign of 'α-excitation' on supramaximal stimulation, even though its afferent fibre was not excited directly. Above, myographic record; below, action potentials on same sweep speed. Stimulus intensity 2·1 times α threshold; a maximal contraction was produced by stimuli 1·3 times α threshold. (The afferent fibre had a threshold to electrical stimulation of 2·3 times α threshold. The A 2 response of the same ending, on much stronger stimulation, is shown in Fig. 2. Initial tension 150 g. No stimulus artifacts present, but the period of stimulation can be judged from the tension records. Records retouched.)

importance it would be expected to overcome the unloading effect of the extrafusal contraction sufficiently to cause the ending to fire during a relatively prolonged isometric tetanic contraction. In 14 out of 27 secondary endings tested the discharge at all initial tensions tested ceased completely during a just-supramaximal tetanic contraction. A typical example is shown in Fig. 11. In 6 other endings the discharge always slowed very markedly and in some of these cases ceased when the initial tension was low. In four endings slowing was only slight, while in three, the discharge accelerated (these were among the 12 endings already discussed). The occurrence of slowing, rather than cessation, of the discharge is no evidence for the existence of α innervation of an ending, for the continued response of the ending during contraction might merely indicate that its resting discharge continued in spite of the small rapid release due to the contraction of the muscle. The only objection to accepting this as the sole explanation was the finding that for two of these endings stimula-

tion at 100/sec caused a slightly greater discharge during the contraction than did stimulation at 50/sec. Such an alteration in stimulation frequency causes only a small increase in the extrafusal contraction (Cooper & Eccles, 1930; Buller, Eccles & Eccles, 1960; Matthews, 1959), but this might be different for different parts of the muscle and thereby cause some muscle spindles to be stretched. The main conclusion is, however, that half of the secondary endings with axons less than 10 μ in diameter are not excited at all by α motor fibres, and no definite evidence was found for a specific α innervation of the remainder.

DISCUSSION

The principal finding of the present investigation is that virtually all muscle spindle afferent endings will give an A 2 response during tetanic contraction of the muscle, provided that the intensity and frequency of stimulation and the initial tension of the muscle are appropriately adjusted. In Matthews's (1933) experiments only a third of the A endings gave an A 2 response, but most of the other A endings could presumably have been induced to do so if a wider range of stimulating conditions had been used. It may be noted that the modern practice of recording the afferent discharge of the ending from a dorsal root filament, rather than from the muscle nerve as Matthews did, largely eliminates the stimulus artifact which otherwise both hinders the detection of weak A 2 responses and discourages the use of any but the lowest frequencies of stimulation. It remains true, however, that under Matthews's particular experimental conditions some endings did and some endings did not give the A 2 response (see also Cooper, 1959). In the present experiments a greater initial tension was required to elicit an A 2 response from secondary endings than from primary endings, and the resulting response was less marked; in addition, A 2 responses could sometimes only be elicited from secondary endings on using moderately high frequencies of stimulation. The significance of these differences between the two kinds of endings is hard to assess, but they are perhaps related to the relatively high threshold of the secondary endings to stretch of the muscle (Hunt, 1954). Since increasing the frequency of stimulation favoured the production of A 2 responses from both types of ending, there is nothing to suggest that they are excited by the contraction of different types of intrafusal muscle fibres with different contractile properties. In any case, the absence of an A 2 response under some particular set of conditions is no guarantee for the absence of an excitatory effect on the ending. For on stimulating the muscle nerve excitation can only be detected when it is great enough to overcome the artificial threshold created both by the unloading effect of the extrafusal

contraction and by the effect on the ending of the antidromic impulses set up by the electrical stimulation of its afferent fibre.

It might be suggested, however, that a crucial feature of Matthews's A 2 responses was that they were set up by stimuli which were only slightly supramaximal for the contraction, while far stronger stimuli were frequently required in the present experiments. Indeed, Hunt & Kuffler (1951*b*) apparently considered that Matthews could not have excited γ fibres with stimuli of this strength, and consequently attributed his A 2 responses entirely to stimulation of ordinary α motor fibres. The question, however, cannot be decided so definitely, since various reasons can be suggested to explain how Matthews's apparently weak stimuli might have excited γ fibres. In the first place, Matthews gave no information about the relative strengths of the stimuli required to elicit threshold contractions and maximal contractions of the muscle; and it is possible that, while the threshold of the most excitable α fibres was normally low the threshold of the least excitable α fibres approached or even overlapped that of the most excitable γ fibres. This could happen if some fibres in the nerve were slightly damaged under the stimulating electrodes, or might be found in a completely normal nerve if the stimulating electrodes were placed very close to the muscle where some α motor fibres would have subdivided into relatively fine branches (Eccles & Sherrington, 1930). In addition, the stimuli used by Matthews were probably of longer duration than those now in common use, and this might have influenced the relative thresholds of α and γ fibres. It is also possible that he placed the stimulating electrodes on or near a region of nerve which had been partly desheathed, for this was a necessary part of his procedure in isolating single fibres. We found, in three preliminary experiments, that desheathing may markedly lower the threshold of the γ fibres relative to that of the α fibres, particularly for stimuli of long duration. Thus it seems reasonable to suggest that Matthews did excite γ fibres with stimuli which were only slightly supramaximal for contraction, because no other explanation is really acceptable for his production of A 2 responses on increasing the strength of stimuli which were already maximal. This interpretation is favoured by the fact that he found sharp thresholds for the production of the A 2 response. We consider, therefore, that most of Matthews's A 2 responses must have been produced by stimulation of γ motor fibres, and demonstrated the excitatory effect on muscle spindle endings of stimulating the motor supply to the intrafusal muscle fibres.

On the other hand it is possible that those of Matthews's A 2 responses which were obtained with stimuli below maximum for the contraction were due to stimulation of α motor fibres. This was observed in a sixth of the present cases on using low-frequency stimulation, and was previously

described by Hunt & Kuffler (1951*b*) using tetanic contractions of shorter duration. We found these effects to be relatively weak and to behave as if mediated by a system with a low 'tetanic fusion frequency', but this would not have prevented their detection in Matthews's experiments, as he usually employed low frequencies of stimulation. They were, however, often absent at low initial tensions and might not have been prominent in his experiments, particularly as his myograph may not have been as rigid as those now in common use. In any case, such excitation is found for both primary and secondary endings and is adequately explained by supposing that some muscle spindles were stretched by the contraction of extrafusal muscle fibres. Any of Matthews's A 2 responses which were due to this type of excitation are, therefore, probably of little importance (Hunt & Kuffler, 1951*b*; Hunt, 1954), but it cannot be accepted that all his A 2 responses were produced in this way. Moreover, his A 2 responses cannot be equated with the 'early discharge' (caused by stimulation of α fibres), as this consists of a very different pattern of firing, and in the one case in which Matthews observed an early discharge he clearly distinguished it from the A 2 response.

During twitch contractions it proved easier to demonstrate for secondary than for primary endings an excitatory effect of using stimuli strong enough to excite γ fibres. This finding is perhaps related to the magnitude of the decrease of the excitability of the ending produced by the release of the muscle spindle occurring during the extrafusal contraction, which might be expected to be greater for the primary ending. For on slowly releasing a stretched muscle the discharge of the primary ending stops immediately, while that of the secondary ending may continue (Cooper, 1959; Harvey & Matthews, unpublished). The difference in behaviour of primary and secondary endings during the twitch might perhaps be explained by their being differently affected by the contractions of different kinds of intrafusal fibres. In view of the complications introduced by the concomitant extrafusal contraction no conclusion on this point can be drawn from the present experiments; but in the cat it seems very improbable that only primary endings can be excited by intrafusal fibres capable of twitches, as has been suggested for the rabbit (Diete-Spiff, 1960). We are uncertain whether the A 2 responses which Matthews described during twitch contractions depended upon γ excitation of the ending, for in the responses illustrated by him the effect is greater than we have seen. They are similar to those described by Hunt & Kuffler (1951*b*) for α excitation, and moreover were produced by increasing the strength of a submaximal rather than of a maximal stimulus. Matthews's classification, however, was apparently based on the behaviour of the endings during tetanic contraction (1933, p. 21), and twitch contraction may not have been

studied sufficiently systematically to see whether complete agreement with the classification could be obtained by these means. The failure of other workers to obtain a clear distinction between A 1 and A 2 responses with twitch contractions would not then be surprising.

It does not now seem possible to decide upon the distribution of primary and secondary endings between Matthews's two groups (cf. Cooper, 1959, 1960). It certainly seems probable that his A 2 group consisted mainly of primary endings, for these give marked A 2 responses on tetanic stimulation; but his A 1 group probably contained some primary endings as well as secondary endings, for he used relatively weak stimuli ('at least' 30 % supramaximal for contraction). Against this view is Matthews's finding that the A 1 endings had a very low threshold to stretch and fired with the least tension on the tendon, while the A 2 endings often had a considerably higher threshold, though never over 10 g (Matthews, 1933, p. 29). The reverse would be expected, as Hunt (1954) found that the threshold to stretch of secondary endings was often much higher than that of the primary endings. Matthews had a few results, based on the relative sizes of action potentials, suggesting that the afferent fibres of A 1 endings were smaller than those of A 2 endings; but his observations were not sufficiently numerous to be applicable to the whole of the series. It may even be questioned whether he studied an appreciable number of secondary endings, as these, unlike the primary endings, are rather insensitive to the rate of application of a stretch (Cooper, 1959), while Matthews's A 1 and A 2 endings were both very sensitive to the rate of stretch. At any rate it seems unlikely that Matthews's division of endings by their A 1 and A 2 behaviour corresponded closely to a division into primary and secondary endings based on measurements of the conduction velocity of the afferent fibres of endings. The classification of muscle spindle endings by their A 1 and A 2 responses therefore seems best discontinued, a view which has already been widely adopted on the basis of the experiments of Hunt & Kuffler (1951*b*).

The fact that all muscle spindle afferent endings may be excited by γ fibre stimulation should not be taken to mean that the γ excitation and control of primary and secondary endings is necessarily identical. The two kinds of endings occupy different positions on the intrafusal muscle fibres, and in initiating the present study we had hoped to confirm a functional difference in the motor control of the endings. We found the A 2 responses of secondary endings weaker and harder to demonstrate than those of primary endings; but both types of endings were excited by stimulation of γ fibres of a wide range of thresholds, and therefore presumably of diameter, and for both primary and secondary endings the excitation appeared to be mediated by a contractile system with a considerably

higher 'fusion frequency' than that of the extrafusal muscle fibres of soleus. Both types of ending were excited on occasion by stimulating α motor fibres alone, and in both cases this could be explained without postulating the existence of a specific α-innervation of the spindle. Thus, though from histological evidence it seems probable that there must be some difference, more fundamental than one of sensitivity, between the responses of primary and secondary endings to intrafusal fibre contraction, it has yet to be demonstrated experimentally.

SUMMARY

1. By recording their discharge from dorsal root filaments the behaviour of de-efferented muscle spindle endings in the soleus muscle of the cat has been studied during tetanic contraction of the muscle elicited by stimulation of the muscle nerve. This was done in order to reinvestigate the classification of muscle spindle afferent endings into two types (Matthews, 1933), according to whether their discharge accelerated (A 2) or slowed (A 1) during supramaximal stimulation.

2. 88 out of 90 endings studied with tetanic stimulation gave an A 2 response when the intensity and frequency of stimulation, and the initial tension of the muscle, were appropriately adjusted. This response was given both by primary and by secondary endings, for the conduction velocities of the afferent fibres studied ranged from 23 to 116 m/sec. If the dividing line of afferent fibre conduction velocity is taken as 72 m/sec (Hunt, 1954), 55 of the endings were primary endings and 35 were secondary endings.

3. A 2 responses could not usually be elicited from secondary endings unless the initial tension was over 50 g, and the tension sometimes had to be considerably higher. Primary endings responded at initial tensions below about 50 g. For both types of endings the A 2 response was favoured by increasing the frequency of stimulation to 70–100/sec, but except for a few secondary endings it was also found on stimulation at 30/sec.

4. The threshold stimulus required to produce an A 2 response was above that required to produce a maximal contraction of the muscle. It was also above the threshold of the most excitable γ motor fibres, which was determined later in the same experiments by recording their compound action potential from a ventral root on stimulating the muscle nerve. The A 2 thresholds of different endings varied considerably, and from a comparison of these values with the thresholds of γ fibres of different conduction velocity it is concluded that both primary and secondary endings may be excited by γ motor fibres of a wide range of diameter.

5. With an initial tension of about 300 g some secondary endings fired

one or two impulses during the rising phase of a twitch, if the stimulus was considerably supramaximal for contraction. At a similar initial tension primary endings did not fire on the rising phase of the twitch contraction, except occasionally when they were so excited by stimulation of α motor fibres.

6. Stimulation of α motor fibres alone (by using weak stimuli) caused, on occasion, two different patterns of excitation of both primary and secondary endings. One was the high-frequency 'early discharge' immediately after the stimulus, while the other discharge was of low frequency. The latter was relatively weak and behaved as if it were mediated by a contractile system with a low 'tetanic fusion frequency'; on stimulation at 70/sec it was masked by the impulses set up by the direct electrical stimulation of the afferent fibre studied and did not interfere with the detection of the A 2 response due to γ excitation of the same ending. The occurrence of such effects does not show whether or not a specific α innervation of the spindle exists for they can be explained otherwise; in any case they were not found for all endings.

7. It is concluded, in agreement with Hunt & Kuffler (1951*b*), that the classification of muscle spindle responses into types A 1 and A 2 lacks simple physiological significance and is best discontinued. It is, however, probable that Matthews's A 2 responses were due to stimulation of γ motor fibres, and demonstrated the effect on muscle spindle endings of the contraction of the intrafusal muscle fibres.

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REFERENCES

BARKER, D. (1948). The innervation of the muscle-spindle. *Quart. J. Micr. Sci.* **89**, 143–186.

BARKER, D. & IP, M. C. (1960). The primary and secondary endings of the mammalian muscle spindle. *J. Physiol.* **153**, 8–10*P*.

BOYD, I. A. (1959). Simple and compound mammalian muscle spindles. *J. Physiol.* **145**, 55–56*P*.

BROWN, M. C. & MATTHEWS, P. B. C. (1960). The effect of a muscle twitch of the back-response of its motor nerve fibres. *J. Physiol.* **150**, 332–346.

BULLER, A. J., ECCLES, J. C. & ECCLES, R. M. (1960). Differentiation of fast and slow muscles in the cat hind limb. *J. Physiol.* **150**, 399–416.

COOPER, S. (1959). The secondary endings of muscle spindles. *J. Physiol.* **149**, 27–28*P*.

COOPER, S. (1960). Muscle spindles and other muscle receptors. In *The Structure and Function of Muscle*. Vol. I, ed. BOURNE, G. H. New York and London: Academic Press.

COOPER, S. & DANIEL, P. M. (1956). Human muscle spindles. *J. Physiol.* **133**, 1*P*.

COOPER, S. & ECCLES, J. C. (1930). Isometric recordings from mammalian muscles. *J. Physiol.* **69**, 377–385.

DIETE-SPIFF, K. (1960). Muscle spindle receptors. *J. Physiol.* **153**, 57*P*.

ECCLES, J. C., ECCLES, R. M. & LUNDBERG, A. (1957). Synaptic actions on motoneurones in relation to the two components of the group I muscle afferent volley. *J. Physiol.* **136**, 527–546.

ECCLES, J. C. & SHERRINGTON, C. S. (1930). Numbers and contraction-values of individual motor-units examined in some muscles of the limb. *Proc. Roy. Soc.* B, **106**, 326–357.

ECCLES, R. M. & LUNDBERG, A. (1959). Synaptic actions in motoneurones by afferents which may evoke the flexion reflex. *Arch. ital. Biol.* **97**, 199–221.

EYZAGUIRRE, C. (1960). The motor regulation of mammalian spindle discharges. *J. Physiol.* **150**, 186–200.

GRANIT, R. (1955). *Receptors and Sensory Perception.* New Haven: Yale: University Press; London: Cumberlege.

GRANIT, R., POMPEIANO, O. & WALTMAN, B. (1959*a*). Fast supraspinal control of mammalian muscle spindles: extra- and intrafusal co-activation. *J. Physiol.* **147**, 385–398.

GRANIT, R., POMPEIANO, O. & WALTMAN, B. (1959*b*). The early discharge of mammalian muscle spindles at onset of contraction. *J. Physiol.* **147**, 399–418.

HARVEY, R. J. & MATTHEWS, P. B. C. (1960). A note on the classification of muscle spindle endings into types A1 and A2. *J. Physiol.* **154**. 25–26*P*.

HUNT, C. C. (1954). Relation of function to diameter in afferent fibres of muscle nerves. *J. gen. Physiol.* **38**, 117–131.

HUNT, C. C. & KUFFLER, S. W. (1951*a*). Further study of efferent small-nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* **113**, 283–297.

HUNT, C. C. & KUFFLER, S. W. (1951*b*). Stretch receptor discharges during muscle contraction. *J. Physiol.* **113**, 298–315.

HUNT, C. C. & PERL, E. R. (1960). Spinal reflex mechanisms concerned with skeletal muscle. *Physiol. Rev.* **40**, 538–579.

HURSH, J. B. (1939). Conduction velocity and diameter of nerve fibres. *Amer. J. Physiol.* **127**, 131–139.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of medullated small-nerve fibres in mammalian ventral roots: efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

LEKSELL, L. (1945). The action potential and excitatory effects of the small ventral root fibres to skeletal muscle. *Acta physiol. scand.* **10**, Suppl. 31.

LLOYD, D. P. C. (1942). Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.* **5**, 153–165.

LUNDBERG, A. & WINSBURY, G. (1960). Selective adequate activation of large afferents from muscle spindles and from Golgi tendon organs. *Acta physiol. scand.* **49**, 155–164.

MATTHEWS, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–53.

MATTHEWS, P. B. C. (1959). The dependence of tension upon extension in the stretch reflex of the soleus muscle of the decerebrate cat. *J. Physiol.* **147**, 521–546.

MERTON, P. A. (1953). Slowly conducting muscle spindle afferents. *Acta physiol. scand.* **29**, 87–88.

RUFFINI, A. (1898). On the minute anatomy of the neuro-muscular spindles of the cat, and on their physiological significance. *J. Physiol.* **23**, 190–208.

WHITTERIDGE, D. (1959). The effect of stimulation of intrafusal muscle fibres on sensitivity to stretch of extraocular muscle spindles. *Quart. J. exp. Physiol.* **44**, 385–393.

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PARTICIPATION BY PRESSURE-PAIN RECEPTORS OF MAMMALIAN MUSCLES IN THE FLEXION REFLEX

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It has been established in an earlier paper (Paintal, 1960) that there are a separate group of sensory endings in muscles that are stimulated by local pressure but not by external stretch. It was suggested that these receptors mediate some kinds of muscle pain; they were therefore termed pressure-pain receptors (Paintal, 1960). Most of these endings are connected to Group III afferent fibres of muscle nerves, some to Group II and a few to Group I fibres. It was also shown that the afferent fibres of Group III terminate mainly in pressure receptors, a few in stretch receptors and some in receptors that cannot be activated mechanically (Paintal, 1960). Like the Group I and Group II bands, therefore, the Group III fibres are a mixed group.

The reflex effects produced by natural stimulation of stretch receptors connected to Group I fibres are well known (Lloyd, 1943*b*; Granit, 1950, 1952; Granit & Ström, 1951; Hunt, 1952) and the results are in agreement with those obtained by stimulating Group I nerve fibres themselves. The effects of natural stimulation of stretch receptors with Group II fibres have been studied in isolation only recently by Laporte & Bessou (1959), who found that the endings of soleus and tibialis anterior respectively inhibit and excite their own motoneurones. So far it has not been possible to study the effects of stimulating endings connected to Group III fibres, since hitherto nothing was known about these endings. However, it is known that electrical stimulation of Group II or Group III fibres facilitates flexor reflexes (Lloyd, 1943*a*; Brock, Eccles & Rall, 1951; Eccles & Lundberg, 1959*a*; Kuno & Perl, 1960), in addition to influencing certain crossed reflexes (Perl, 1958). Since it is now known that the majority of Group III fibres terminate in pressure-pain receptors (Paintal, 1960) it is to be expected that the reflex effects of these endings will be essentially similar to the effects of stimulating Group III fibres—an expectation borne out by the results of the present investigation, which was aimed at determining the reflex effects of pressure-pain receptors of triceps surae and tibialis anterior.

METHODS

Adult cats were anaesthetized with ether and the spinal cord was cut at the level of the atlanto-occipital membrane. The brain was destroyed by pithing and the animal was put on artificial ventilation. The pelvis and the left hind limb were immobilized by transfixing them with pins, and muscular movements were prevented by injecting D-tubocurarine or gallamine (Flaxedil; May & Baker) intravenously. The sural and tibial nerves were always cut; the latter was cut distal to its branches to the triceps surae as far centrally as possible, to avoid stimulating its central end when the intact lateral gastrocnemius-soleus nerve was stimulated. Other nerves were cut according to the nature of the experiment.

In some experiments local pressure was applied to the triceps surae muscle by an electromechanical device (presser) powered by an audioamplifier into which square pulses of short duration were fed from a stimulator triggered by the sweep of the oscilloscope. In these experiments the flattened end of a wooden rod connected to the presser was applied to the ventral surface of the triceps surae, which was exposed by separating this part of the muscle from the underlying bone. The tendo Achillis was elevated after cutting the plantaris tendon and the muscle was sandwiched between the rod of the presser and a pressure gauge applied to its dorsal surface. The part so sandwiched consisted of the muscular part of the muscle, about 2–3 cm from the point of insertion of the tendon on the calcaneus. In these experiments the intensity and duration of the pressure pulses were kept constant. Usually about 0·5–1 kg pressure was applied and the duration of the pressure pulse was about 3·5 msec (Fig. 4). Often, as in Fig. 4*B*, there were some oscillations of low amplitude after the main pressure pulse. These should be kept in mind when considering the conditioning effects of pressure pulses applied at large intervals before the test stimulus. The pressure gauge was connected to a d.c. amplifier and led to one channel of the oscilloscope; it was calibrated by applying weights to its surface.

Monosynaptic reflexes were recorded monophasically from the cut central end of L 7 or S 1 ventral roots. These reflexes were elicited by stimulating the cut central ends of the following nerves: posterior biceps semitendinosus (BST), triceps surae, deep peroneal (DP) and tibialis anterior (TA). Occasionally an intact nerve was stimulated in order to avoid cutting off the sensory inflow from a particular muscle. The conduction time for the Group I volley from the stimulating electrodes on a peripheral muscle nerve to the spinal cord was determined at the end of an experiment by recording the arrival of the afferent volley monophasically from the peripheral end of a cut dorsal root near its entry into the spinal cord.

Whenever it was necessary to take many records of monosynaptic reflexes, the oscilloscope trace was masked except for the section occupied by the reflex (Figs. 2, 3 and 7). This procedure allowed continuous records to be taken over prolonged periods with considerable economy of recording paper.

RESULTS

Stimulation of pressure-pain receptors in lateral gastrocnemius and soleus (LGS)

Effects on posterior biceps and semitendinosus (BST) monosynaptic reflex. Since many pressure-pain receptors of lateral gastrocnemius and soleus are located near the junction between the muscle and the tendo Achillis (Paintal, 1960), this region was chosen exclusively for stimulating these receptors; it was usually situated about 2·5–3 cm central to the insertion of the tendon on the calcaneus. This was a fortunate choice, because it was found later that squeezing this part caused little stimulation of stretch

receptors, as revealed by recording impulses from the whole lateral gastrocnemius-soleus (LGS) nerve (Fig. 1).

Squeezing the muscle either between finger and thumb or between the jaws of dissecting forceps always increased the BST monosynaptic reflex (Fig. 2). This response survived the cutting of all nerves to the hind limb with the exception of the nerve to the lateral gastrocnemius and soleus. To distinguish the action of pressure-pain receptors from those of stretch

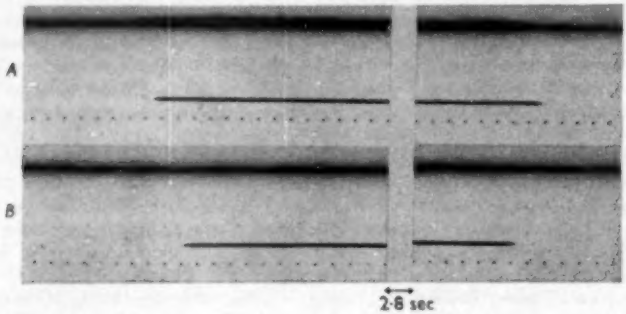

Fig. 1. Records of impulse activity in peripheral end of cut lateral gastrocnemius-soleus nerve, *A* while stretching the muscle, and *B* while squeezing the musculotendinous region, during signals. From above downwards in each record, impulse activity, signal and 1/10 sec time marks.

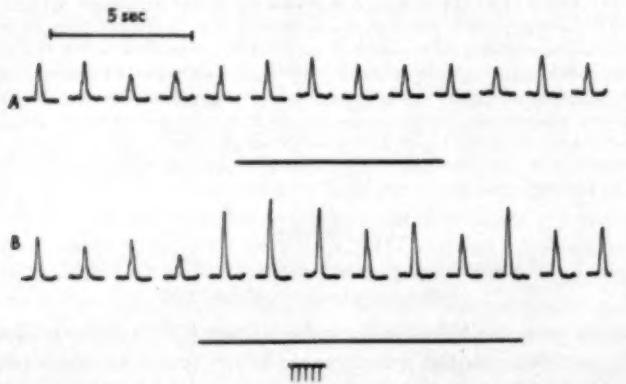

Fig. 2. Segments of sweeps showing biceps-semitendinosus (BST) monosynaptic responses. The sweeps recur at intervals of 1·6 sec and were taken on moving recording paper. During signal in *A* the triceps surae was pulled, and in *B* it was squeezed between finger and thumb. Medial gastrocnemius nerve was intact. Note increase in monosynaptic response while muscle was squeezed. Time marker, msec.

receptors, which, as mentioned above, can also be stimulated a little by squeezing the muscle, the effects of local pressure were compared with those of stretching the muscle. Since it is known that Group I fibres (Eccles, Eccles & Lundberg, 1957) and Group II fibres (Kuno & Perl, 1960) of triceps surae facilitate BST motoneurones, it was not surprising to find that stretching the muscle to about 700 g often facilitated these motoneurones. However, this facilitation was very often much less than that produced by squeezing the muscle. Typical responses are shown in Fig. 2. When these are considered in relation to the impulses produced by stretching and squeezing the muscle (Fig. 1) it may be concluded that squeezing the muscle facilitates the reflex primarily by stimulating endings other than stretch receptors, probably pressure-pain receptors (Paintal, 1960).

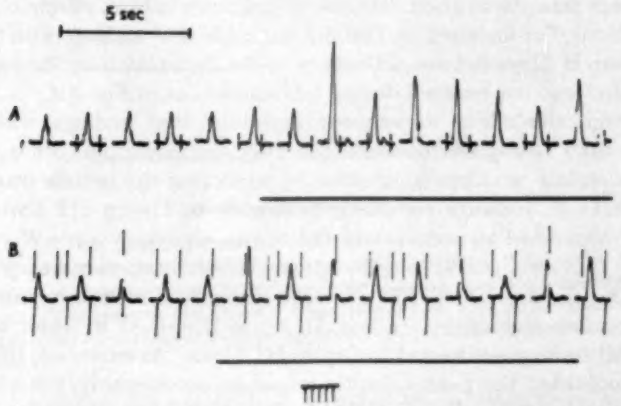

Fig. 3. BST monosynaptic responses. Record *A* was taken while the LGS nerve was stimulated repetitively at 270/sec with stimuli 10 times threshold for Group I. In *B* the strength of the stimuli was 66 times threshold. In both *A* and *B* the triceps surae was squeezed during signals. Note that the effect of pressure is not blocked by 10 times threshold stimuli in *A*. The sweeps recur every 1·6 sec. Time marker, msec.

In order to show that pressure-pain endings with Group III fibres (or smaller) facilitate the reflex, the receptors connected to Group I and Group II fibres were depressed antidromically by repetitive stimulation at a frequency ranging from 200 to 460/sec. Since Eccles & Lundberg (1959*b*) have shown that all Group II fibres should be stimulated by stimuli 8–10 times the threshold for Group I, stimuli of at least this strength were used to stimulate the intact LGS nerve. However, in most experiments stimuli of about 16 times threshold were used, to allow for any relative refractoriness in the nerve fibres at high frequencies of stimulation. These stimulus

strengths have been used throughout this investigation to stimulate Group I and Group II fibres. It is to be expected from the observations of Eccles & Lundberg (1959*b*) and Kuno & Perl (1960) that at this stimulus strength some Group III fibres will also be excited. This does not vitiate the results, since the main purpose of these experiments was to determine the reflex effects of endings connected to Group III fibres only.

As had been expected, tetanic stimulation by itself enhanced the BST monosynaptic responses initially but the responses soon stabilized themselves (see Eccles & Rall, 1951). While the nerve was thus tetanized it was found that facilitation of BST monosynaptic reflex by squeezing the muscle was not obviously reduced (Fig. 3*A*), thus showing that endings with fibres smaller than Group II facilitate the reflex by pressure. No conclusion can, however, be drawn about the role of Group I and Group II fibres from this observation, because of unknown central effects of tetanic stimulation. For instance, one cannot conclude that endings with Group I and Group II fibres did not contribute to the facilitation on the basis that facilitation was not reduced during tetanization as in Fig. 3*A*.

Although the above experiment indicated that endings with fibres smaller than Group II facilitate the BST monosynaptic reflex, it was still not certain whether facilitation by squeezing the muscle was due to stimulation of pressure receptors connected to Group III fibres or to endings connected to non-myelinated fibres, especially since Voorhoeve, Laporte & Bessou (1958) have demonstrated facilitation of flexor reflexes by non-myelinated fibres. The stimulus strength of repetitive stimulation was therefore increased to about 70 times threshold in order to block nearly all endings connected to Group III fibres. As expected, this sometimes facilitated the monosynaptic responses considerably, but after they had become stabilized it was found that facilitation by squeezing the muscle was much reduced (Fig. 3*B*), although it was still present in some experiments; this was presumably due to endings connected to non-myelinated fibres. No definite conclusion concerning the relative contribution by pressure-pain endings with Group III fibres can be drawn from this experiment, because the reduced facilitation during tetanization, shown in Fig. 3*B*, may have been due entirely to the central effects of tetanic stimulation.

Evidence that pressure-pain receptors connected to Group III fibres facilitate the BST monosynaptic reflex was obtained by applying pressure pulses to the triceps surae with the presser as described in methods. This procedure itself yielded a reflex discharge in ventral roots with a latency ranging from 15 to 28 msec (Fig. 4*B*), but since the destination of the motoneurones stimulated could not be ascertained this aspect of the response was not pursued further.

As shown in Fig. 4*B*, the facilitation of the BST monosynaptic response by pressure pulses was marked, being sometimes three times greater than the control responses. The interval between the pressure pulse and the BST test stimulus at which facilitation by the pressure pulses appeared varied from 12 to 18 msec. Maximum facilitation appeared at about 26–30 msec. Since the central delay associated with the facilitation of the reflex by the larger LGS afferent fibres is small (see Kuno & Perl, 1960), these long latencies suggest that slowly conducting myelinated fibres were responsible for the facilitation, assuming that the excitation time of the endings by the pressure pulses did not take more than 1–3 msec. However, participation by pressure-pain and stretch receptors connected to Group I and Group II fibres could not be ruled out in such an experiment and the

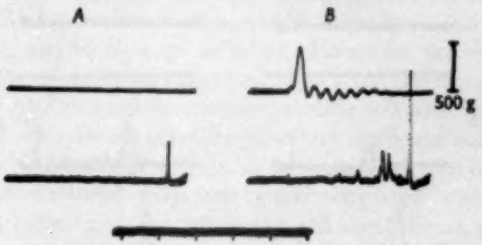

Fig. 4. Effect of pressure pulses to triceps surae on BST monosynaptic responses. The lower traces in *A* and *B* show the monosynaptic response. *B* shows that application of a pressure pulse (upper trace) greatly increases the monosynaptic response. The pressure also yields a reflex response in some unidentified motoneurones before the flexor monosynaptic reflex in *B*. Time marker, msec and 10 msec.

LGS nerve was therefore tetanized as described earlier, in order to depress antidromically all endings connected to these larger fibres. While the nerve was thus tetanized the effect of pressure was examined and it was found that facilitation by the pressure pulses though reduced was still prominent (Fig. 6). These results therefore prove that pressure receptors connected to Group III fibres facilitate the BST monosynaptic reflex.

The contribution through the larger fibres was excluded also by applying single volleys of about ten times threshold simultaneously with the pressure pulse. It was confirmed by recording impulses from the cut peripheral end of the whole LGS nerve that this single volley considerably reduced the observable discharge in large fibres. As is shown in Fig. 5*D*, this produced no change or only slight reduction in the facilitation produced by the pressure pulse. This shows that considerable reduction of the afferent input through the larger fibres had no effect on the facilitation by pressure pulses. In this experiment it was necessary to ensure that the

interval between the LGS stimulus and the test BST stimulus was such that the first stimulus did not itself alter the response. To be on the safe side, the moment of its application (and therefore also that of the pressure pulse) was so adjusted that it tended to reduce the monosynaptic reflex (compare Fig. 5*A* with 5*B*). The unobservable central effects of the LGS stimulus must be kept in mind when interpreting the changes quantitatively, and since these effects are unknown no attempt has been made to evaluate the relative contribution by the large fibres to the total facilitation shown in Fig. 5*C*.

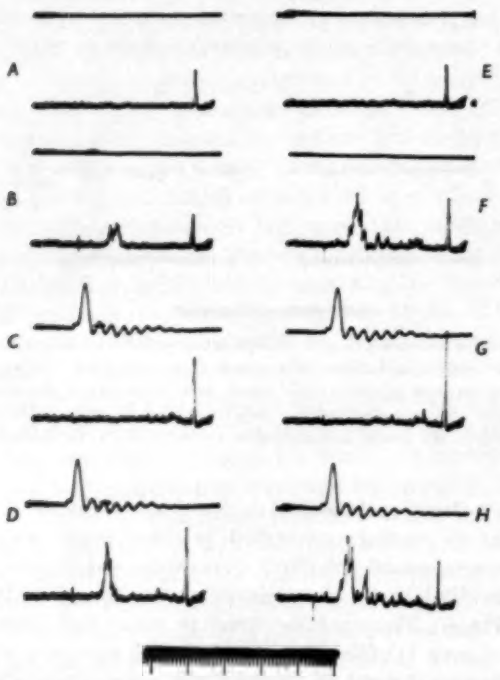

Fig. 5. BST monosynaptic reflex. *A* and *E* show the size of the control response (lower traces). *C* and *G* show the effect of pressure pulses on this response. In *B* a 10-times-threshold stimulus to the intact LGS nerve was applied before the flexor stimulus at such an interval that it did not increase the response. In *D* the stimulus and pressure were applied together; the undiminished response, compared to those in *C* and *G*, shows that the 10-times-threshold stimulus did not block the facilitating effect of the pressure pulse. In *F* the stimulus was 49 times threshold, i.e. sufficient to stimulate most Group III fibres. Application of this stimulus and the pressure pulse together in *H* prevented the facilitatory effect of the pulse. Time marker, msec and 10 msec.

It was thought that if the strength of the LGS stimulus was increased to about sixty times threshold, impulses from endings from Group III fibres would also be blocked. This was done, as shown in Fig. 5*F*, *G* and *H* and it was found that facilitation by the pressure pulses was prevented (Fig. 5*H*). This was interpreted to mean that the strong stimulus prevented BST facilitation by depressing the endings of Group III fibres peripherally, because care was taken to ensure that the stimulus did not itself reduce the monosynaptic reflex by adjusting its position and therefore also that of the pressure pulse so that the LGS stimulus in fact facilitated the monosynaptic response a little (compare Fig. 5*E* and *F*). However, this interpretation is not unequivocal, because it is possible that the central effects of the LGS stimulus itself may have been responsible for preventing the facilitation. A second possibility, that the strong LGS stimulus stimulated the same BST motoneurones that would have been facilitated by the pressure pulse, has to be kept in mind. However, the reflex discharge produced by the stronger stimulus itself was often not much greater than that produced by the weaker one (compare Fig 5*D* with 5*H*).

The interval between the pressure pulse and the flexor volley was varied and the effect of this on the monosynaptic responses was noted. At each interval ten conditioned responses were compared with twenty test responses (ten before and ten after) and the conditioned responses were expressed as a percentage of the test responses. As expected the facilitation varied with the interval between the pulse and the flexor volley (Fig. 6). Figure 6 shows that, apart from the reduction in the facilitation, there is not much difference in the curves obtained with and without tetanization of the LGS nerve. Since the minimum latency for facilitation is also the same, this indicates that in this particular experiment, endings connected to Group I and Group II fibres did not contribute significantly to the facilitation by pressure in the absence of tetanic stimulation. The reduced facilitation during tetanic stimulation could be due to the central effects of tetanic stimulation or to the antidromic stimulation of some Group III fibres. Since for facilitation to occur the minimum interval between the initiation of impulses by the pressure pulse (allowing about 3 msec for initiation time at the endings) and the arrival of the flexor volley at the spinal cord is about 15 msec, and since the conduction distance from the point of application of pressure to the spinal cord was about 220 mm, it follows that the volley of impulses were conducted over fibres with conduction velocity of at least 15 m/sec. As the interval was increased, impulses over more slowly conducting fibres could also exert their effects and thus add to the facilitation as shown in Fig. 6.

In the experiment from which the graph of Fig. 6 was plotted it was found that ten-times-threshold conditioning volleys (adequate to stimulate

all Group II fibres (Eccles & Lundberg, 1959*b*)) did not facilitate the BST monosynaptic reflex, whereas there was obvious facilitation with stimuli of greater intensity. This was not the common type of response, but it provides evidence to show why receptors connected to larger fibres did not contribute to the facilitation by pressure pulses in this experiment. In most of the experiments, however, there was obvious facilitation of BST monosynaptic reflex with stimuli less than twice threshold. Indeed the

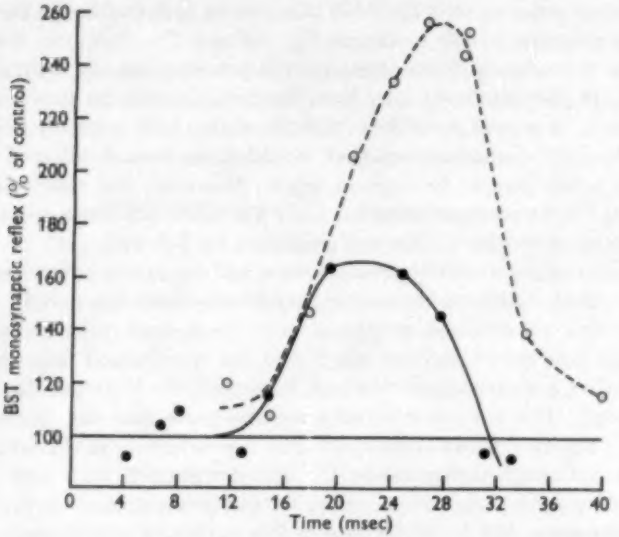

Fig. 6. Conditioning effect of pressure pulses to triceps surae on BST monosynaptic reflex (ordinate). The abscissa represents the interval between the application of the pressure pulse and the time of arrival of the flexor Group I volley at the spinal cord. The ordinates indicate the size of the monosynaptic response expressed as a percentage of the control response. Graph - -○- -○- - shows responses without tetanization of LGS nerve; graph —●—●— shows responses while the nerve was tetanized continuously at 460/sec with stimuli 13 times threshold for Group I.

curves showing the conditioning effect of LGS volleys on BST monosynaptic responses were similar to those obtained by Kuno & Perl (1960) and to those obtained by Eccles & Lundberg by applying conditioning stimuli to the plantaris nerve in the spinal preparation (Eccles & Lundberg, 1959*a*). In such experiments, unlike that shown in Fig. 6, the latency at which facilitation by pressure set in while the LGS nerve was tetanized was clearly greater than when the nerve was not tetanized. For example, in one experiment without tetanization the latency was 12 msec

and with tetanization 20 msec. This suggests that endings connected to larger fibres, probably those of Group II, were responsible for the earlier facilitation. This is understandable, because stretch receptors with Group II fibres may be stimulated by the pressure pulse and there are significant numbers of pressure-pain receptors connected to Group II fibres (Paintal, 1960).

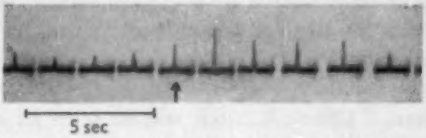

Fig. 7. Record to show the effect on BST monosynaptic reflex of introducing a hypodermic needle at arrow into triceps surae near tendo Achillis. The sweeps recur every 1·6 sec.

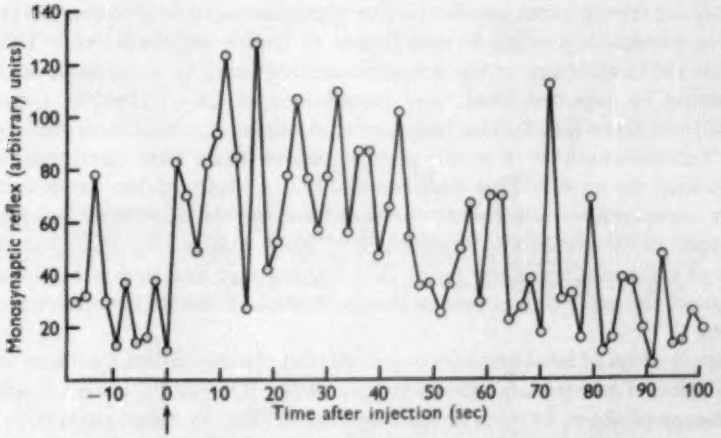

Fig. 8. Graph to show the effect on the BST monosynaptic reflex of injecting 0·5 ml. 6 % NaCl into the triceps surae at arrow.

All the foregoing experiments have shown that pressure-pain receptors of LGS facilitate BST monosynaptic reflex. Since these receptors are stimulated by introducing a hypodermic needle into the muscle or by injecting 6 % NaCl solution locally (Paintal, 1960), it is expected that these procedures will also facilitate BST motoneurones, which in fact they most often did. The facilitation produced by introducing a needle was usually short-lasting (Fig. 7). This is to be expected, because this procedure only yields a short train of impulses from pressure-pain receptors (Paintal, 1960). The facilitation following local injection of 0·5 ml. 6 % NaCl set in

within a few seconds and it often persisted for 2–3 min (Fig. 8). This is to be expected from the effects of 6% NaCl on pressure-pain receptors. The contribution by endings connected to larger fibres may be ignored, because local injection of 6% NaCl at the peripheral site chosen for injection (i.e. near the musculo-tendinous junction) usually has no noticeable effect on stretch receptors (Paintal, 1960). However, rarely a massive discharge in the larger fibres may be produced by 6% NaCl, so that facilitation of the BST monosynaptic reflex through Group I and Group II fibres had to be ruled out. This was done by tetanic stimulation, as described earlier, and it was found that the facilitatory effect of 6% NaCl persisted while the LGS nerve was tetanized. Although these results once again indicate that pressure-pain receptors with Group III fibres facilitate the BST monosynaptic reflex, the possible contribution by endings connected to non-myelinated fibres must be kept in mind.

Effects on triceps surae motoneurones. It has been confirmed that strongly stretching triceps surae can inhibit the triceps surae or LGS monosynaptic reflex, presumably owing to stimulation of tendon organs (Granit, 1950; Hunt, 1952), although in the initial phase there may be some facilitation, as would be expected from the observations of Lloyd (1943*b*), Granit (1950) and Hunt (1952). On the other hand, squeezing the muscle inhibited the motoneurones to a much greater extent than that produced by stretching the muscle. This was presumably due to stimulation of pressure-pain receptors, a conclusion strengthened by the fact that inserting a hypodermic needle and injecting 6% NaCl locally also inhibited the triceps surae monosynaptic reflex; the latency and duration of inhibition followed the same time course as the facilitation of the BST monosynaptic reflex.

Application of local pressure pulses yielded similar results, i.e. there was inhibition of triceps surae monosynaptic reflex. This inhibition set in after a latency of about 20 msec in one experiment (Fig. 9), a fact suggestive of action mediated by slowly conducting myelinated fibres. In the experiment illustrated in Fig. 9 the inhibition became so pronounced that at an interval greater than 29 msec the monosynaptic reflex was completely inhibited by the pressure pulse. In these experiments the LGS nerve was intact and test stimuli were applied either to this nerve alone or to both this and the central end of the cut medial gastrocnemius nerve.

Effects on tibialis anterior (TA) or deep peroneal (DP) monosynaptic reflex. Stretching triceps surae always inhibited TA or DP monosynaptic responses, as would be expected from the observations of Granit (1952). On the other hand squeezing triceps surae occasionally either slightly facilitated these motoneurones or was without effect. In these cases conditioning stimuli of Group III strength to LGS nerve clearly facilitated the

DP monosynaptic reflex. This is in agreement with some of the observations of Eccles & Lundberg (1959*b*). More frequently, however, squeezing triceps surae inhibited the DP monosynaptic reflex markedly (Fig. 10) and correspondingly, Group III strength conditioning volleys to cut LGS nerve produced pronounced inhibition. This inhibition by squeezing triceps surae is surprising, because pretibial muscles are flexors and Group III fibres

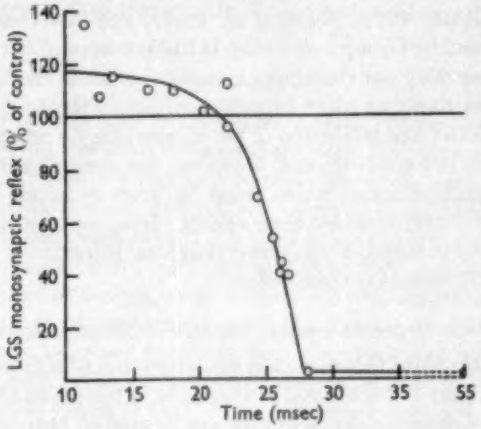

Fig. 9. Graph showing conditioning effect of pressure pulses applied to triceps surae on LGS monosynaptic reflex. Abscissa indicates interval between the application of pressure pulse and arrival of Group I LGS volley at spinal cord. Ordinate represents size of monosynaptic response expressed as percentage of control response.

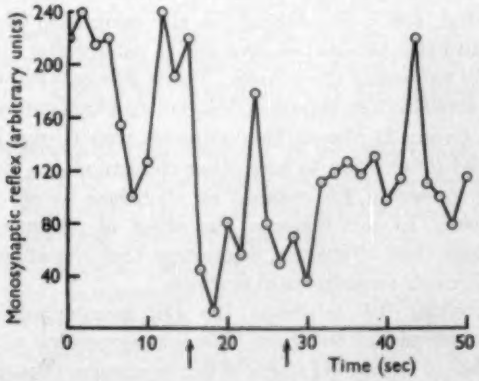

Fig. 10. Graph showing inhibitory effect of squeezing triceps surae, between arrows, on deep peroneal monosynaptic reflex, expressed in arbitrary units.

(which end predominantly in pressure-pain receptors (Paintal, 1960) of muscle nerves are known to facilitate the flexion reflex (Lloyd, 1943*a*; Eccles & Lundberg, 1959*a*, *b*). The evidence that this inhibition is caused by pressure-pain receptors is very strong, because stimulation of tendon organs (which are more likely to be stimulated than muscle spindles by squeezing the musculo-tendinous junction of triceps surae) of LGS or their nerve fibres facilitates DP monosynaptic reflexes (Laporte & Lloyd, 1952; Granit, 1952; Hunt, 1952; Eccles *et al.* 1957). The fact that these effects were not mediated by Group I or Group II fibres was established, as already described, by carrying out the above manoeuvres while the LGS nerve was stimulated repetitively to block impulse activity in Group I and Group II fibres. As expected, the inhibition of DP monosynaptic reflex by squeezing triceps surae was not much altered. However, the possible effects of endings connected to unmyelinated fibres must be kept in mind, because these experiments have not excluded their effects. In agreement with the effects of squeezing triceps surae, it was noted that local injection of 6 % NaCl also inhibited the DP monosynaptic reflex.

Effects of pressure-pain receptors in tibialis anterior

In order to stimulate pressure-pain receptors, the tibialis anterior muscle was squeezed about 1–2 cm distal to the entry of the nerve into the muscle, because many pressure-pain receptors are located in this region (Paintal, 1960). This produced much less facilitation of BST monosynaptic reflexes than that produced by squeezing triceps surae; sometimes there was no facilitation at all. In view of the current belief that Group III fibres of muscles facilitate flexor reflexes, this behaviour was quite unexpected, because there are apparently more pressure-pain receptors in TA than there are in LGS (Paintal, 1960). Stretching TA also facilitated the BST monosynaptic reflex and this facilitation was either equal to or little less than that produced by squeezing the muscle. These effects, although reduced, survived during repetitive stimulation designed to block endings connected to Group I and Group II fibres. This indicates that Group III fibres are probably involved in addition to any other concerned.

Stretching or squeezing TA reduced or abolished triceps surae monosynaptic responses. In this instance the effect of pressure was clearly more marked than that of stretch, suggesting that the effect of pressure was mediated through pressure-pain endings.

Whereas stretching TA inhibited the DP monosynaptic responses, squeezing the muscle facilitated them. This is in agreement with the known effects of stimulating Group III fibres of flexor muscles (Brock *et al.* 1951). Sometimes squeezing the muscle inhibited DP motoneurones, an effect presumably attributable to the stretch receptors because squeezing TA,

unlike squeezing triceps surae, yields an appreciable discharge in stretch receptors.

Stretching or squeezing extensor digitorum longus muscle about its middle produced effects similar to those obtained from TA. Since there are certain similarities in the shape and size of these two muscles it is probable from these results that there are many pressure-pain receptors located somewhere near the middle of this muscle also.

DISCUSSION

The main conclusions to be drawn from these results are that impulses from pressure-pain receptors connected to Group III fibres of LGS facilitate the BST monosynaptic reflex and inhibit their own, and although they sometimes facilitate those of pretibial muscles they more often inhibit them; pressure-pain receptors of TA (and probably also those of extensor digitorum longus) facilitate their own motoneurones and those of BST and they inhibit those of triceps surae. With the exception of one observation all the others are in conformity with the known effects of stimulating Group III fibres, i.e. they facilitate flexor muscles and inhibit extensors in accordance with the pattern of the flexion reflex (Lloyd, 1943*a*; Eccles & Lundberg, 1959*a*; Kuno & Perl, 1960). However, pressure-pain receptors of different muscles are not equally effective in facilitating the flexion reflex, because facilitation of BST monosynaptic responses by LGS pressure-pain receptors is sometimes several times greater than that produced by TA receptors in the same experiment, in spite of the fact that there are apparently more pressure-pain receptors in TA than in LGS (Paintal, 1960). The results also fit in with those of Brock *et al.* (1951), because the pressure-pain receptors of extensors (triceps surae) inhibit their own, and those of flexors facilitate their own, motoneurones. This is what Brock *et al.* referred to as autogenetic inhibition and facilitation by Group III fibres.

The only observation that does not fit into the general scheme is the inhibition of TA or DP monosynaptic reflexes by pressure-pain receptors of LGS or by conditioning stimuli of Group III strength to the nerves of triceps surae. At first this was somewhat perplexing, but when the observations of Eccles & Lundberg (1959*b*) were published the reason for this behaviour became understandable. Eccles & Lundberg have shown that volleys to Group III fibres of LGS can produce both excitatory and inhibitory post-synaptic potentials in DP motoneurones and that mixed effects are often in evidence in the same motoneurone. This they explained by suggesting that there are two pathways from high-threshold afferent fibres to flexor motoneurones—the excitatory one open normally in spinal

cats and the inhibitory one functioning when the excitatory one is suppressed, e.g. by low blood pressure (Eccles & Lundberg, 1959*b*) or by suitable accessory stimulation (Kuno & Perl, 1960). However, this state of affairs applies to only certain muscles, because there are other muscles, e.g. BST which are not inhibited by pressure-pain receptors—this being in agreement with the observations of Eccles & Lundberg (1959*b*).

Tibialis anterior is probably an exceptional flexor in that it is made up of a mixture of slow and fast fibres, the latter occupying the more superficial part of the muscle (Gordon & Phillips, 1953). These subdivisions also differ in their reflex responses because Gordon & Phillips (1953) noted that the deeper motor units had a lower reflex threshold in response to pinching the toes and the superficial ones a higher maximal frequency of firing in flexion reflexes. These differences must be kept in mind when interpreting reflex changes in the monosynaptic reflex of TA in response to stimulation of pressure-pain receptors.

If the DP motoneurones were the only flexor motoneurones that yield mixed responses to stimulation of pressure-pain receptors or their Group III fibres, one could, for the time being, ignore this solitary evidence that does not fit into the pattern of the flexion reflex. However, some other observations of Eccles & Lundberg (1959*a*) indicate that this is not the only exception, because Group III fibres of quadriceps can also inhibit semitendinosus motoneurones. This inhibition, which apparently appears at higher stimulus strengths, is not due to Renshaw inhibition (Eccles & Lundberg, 1959*a*). The authors suggested once again that this could be due to the existence of two pathways by which impulses in high-threshold muscle afferent fibres could act on flexor motoneurones.

The reflex effects of pressure-pain receptors connected to Group II fibres have not been studied in detail because it was not possible to block all fibres other than those of Group II. On the other hand, while studying the effects of Group III pressure-pain receptors it was possible to block by repetitive antidromic stimulation all the endings connected to Group I and Group II fibres. An important shortcoming of this method of blocking conduction of impulses in selected fibres is that the central effects of repetitive stimulation may be considerable in magnitude, and are largely unknown. This criticism must be kept in mind even though in this investigation the nerve used for eliciting the monosynaptic reflex was not tetanized and the reflex effects of natural stimuli were examined only after the monosynaptic responses had become stabilized following the onset of tetanic stimulation (see Fig. 3). However, in spite of its limitations it can provide valuable information if the results are interpreted conservatively, as has been done in this investigation. Besides, no other method (e.g. pressure, temperature, electrotonic block or local anaesthesia)

offers as precise a means of producing a graded and relatively constant block of impulses from sensory endings for prolonged periods.

Although the results of the present investigation have provided good agreement between the effects of natural stimulation and the effects of nerve volleys, it must be pointed out that the interpretation of the effects of the latter have become complicated, since it is now known that Group II fibres innervate a heterogeneous group of stretch and pressure-pain receptors (Paintal, 1960). In addition, some pressure-pain receptors are also connected to Group I fibres so that there are functionally three kinds of fibres in Group I. As was pointed out by Lloyd (1957), the effects of Group II fibres connected to stretch receptors cannot be regarded as nociceptive and therefore the customary practice of lumping together the reflex effects of Group II and Group III fibres, the latter being mostly connected to pressure-pain receptors, should perhaps be abandoned.

SUMMARY

1. The posterior biceps semitendinosus (BST) monosynaptic reflex was conditioned by natural stimulation of sensory receptors of muscles and it was shown that squeezing the triceps surae near the tendo Achillis or the tibialis anterior facilitated this reflex in spinal cats.

2. By applying pressure pulses locally and by blocking the endings of selected afferent fibres by stimulating them either tetanically or by single volleys, it was established that pressure-pain receptors connected to Group III afferent fibres facilitate the BST monosynaptic reflex. There was suggestive evidence that this reflex was also facilitated by endings connected to non-myelinated fibres.

3. Stimulating pressure-pain receptors of LGS inhibited triceps surae monosynaptic reflex and it sometimes facilitated DP monosynaptic reflex in accordance with the pattern of the flexion reflex. More frequently, however, it inhibited DP or TA monosynaptic reflexes markedly. This peculiarity has been discussed in relation to the findings of previous investigators.

4. The results obtained show that stimulation of pressure-pain receptors connected to Group III fibres facilitates the flexion reflex and are in conformity with those obtained by stimulating the afferent fibres themselves.

I am indebted to Professor C. C. Hunt, Dr E. R. Perl and Professor A. Lundberg for valuable criticisms.

REFERENCES

BROCK, L. G., ECCLES, J. C. & RALL, W. (1951). Experimental investigations on the afferent fibres in muscle nerves. *Proc. Roy. Soc.* B, **138**, 453–475.

ECCLES, J. C., ECCLES, R. M. & LUNDBERG, A. (1957). Synaptic actions on motoneurones caused by impulses in golgi tendon organ afferents. *J. Physiol.* **138**, 227–252.

ECCLES, R. M. & LUNDBERG, A. (1959*a*). Supraspinal control of interneurones mediating spinal reflexes. *J. Physiol.* **147**, 565–584.

ECCLES, R. M. & LUNDBERG, A. (1959*b*). Synaptic actions in motoneurones by afferents which may evoke the flexion reflex. *Arch. ital. Biol.* **97**, 199–221.

ECCLES, J. C. & RALL, W. (1951). Repetitive monosynaptic activation of motoneurones. *Proc. Roy. Soc.* B, **138**, 475–498.

GORDON, G. & PHILLIPS, C. G. (1953). Slow and rapid components in a flexor muscle. *Quart. J. exp. Physiol.* **38**, 35–45.

GRANIT, R. (1950). Reflex self-regulation of the muscle contraction and autogenetic inhibition. *J. Neurophysiol.* **13**, 351–372.

GRANIT, R. (1952). Reflexes to stretch and contraction of antagonists around ankle joint. *J. Neurophysiol.* **15**, 269–279.

GRANIT, R. & STRÖM, G. (1951). Autogenetic modulation of excitability of single ventral horn cells. *J. Neurophysiol.* **14**, 113–132.

HUNT, C. C. (1952). The effect of stretch receptors from muscle on the discharge of motoneurones. *J. Physiol.* **117**, 359–379.

KUNO, M. & PERL, E. R. (1960). Alteration of spinal reflexes by interaction with suprasegmental and dorsal root activity. *J. Physiol.* **151**, 103–122.

LAPORTE, Y. & BESSOU, P. (1959). Modifications d'excitabilité de motoneurones homonymes provoquées par l'activation physiologique de fibres afférents d'origine musculaire du groupe II. *J. Physiol., Paris*, **51**, 897–908.

LAPORTE, Y. & LLOYD, D. P. C. (1952). Nature and significance of the reflex connections established by large afferent fibers of muscular origin. *Amer. J. Physiol.* **169**, 609–621.

LLOYD, D. P. C. (1943*a*). Neuron patterns controlling transmission of ipsilateral hind limb reflexes in cats. *J. Neurophysiol.* **6**, 293–315.

LLOYD, D. P. C. (1943*b*). Conduction and synaptic transmission of the reflex response to stretch in spinal cats. *J. Neurophysiol.* **6**, 317–326.

LLOYD, D. P. C. (1957). Input–output relation in a flexor reflex. *J. gen. Physiol.* **41**, 297–306.

PAINTAL, A. S. (1960). Functional analysis of Group III afferent fibres of mammalian muscles. *J. Physiol.* **152**, 250–270.

PERL, E. R. (1958). Crossed reflex effects evoked by activity in myelinated afferent fibres of muscle. *J. Neurophysiol.* **21**, 101–112.

VOORHOEVE, P. E., LAPORTE, Y. & BESSOU, P. (1958). A flexor reflex provoked by stimulation of non-medullated C-fibres in the cat. *Acta physiol. pharm. neerl.* **8**, 299–300.

J. Physiol. (1961), **156**, *pp.* 515–522
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THE STIMULANT EFFECT OF BARIUM ON THE RELEASE OF ACETYLCHOLINE FROM THE SUPERIOR CERVICAL GANGLION

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Calcium and magnesium are known to influence the quantity of acetylcholine (ACh) that is released by impulses in cholinergic nerves. Thus the output of acetylcholine from the superior cervical ganglion during stimulation of the preganglionic trunk is reduced when the perfusion medium is deficient in calcium (Harvey & MacIntosh, 1940) or when it contains excess magnesium, and is increased when the medium contains calcium in excess of the usual amount (Hutter & Kostial, 1954); and there is electrophysiological evidence indicating that these two ions have similar antagonistic effects on the release of acetylcholine from motor nerve endings in skeletal muscle (del Castillo & Katz, 1956).

The present paper offers evidence that barium, a divalent ion closely related to calcium and magnesium, also influences acetylcholine release from cholinergic nerves. A preliminary account of the findings was presented at the meeting of the Physiological Society in Lund last July (Douglas, Lywood & Straub, 1960).

METHODS

Preparation. Cats were anaesthetized with chloralose, 80 mg/kg, I.V., following induction with ethylchloride and ether. One or other superior cervical ganglion was prepared for perfusion after the method of Kibjakow (1933) with the modifications described by Perry (1953). The whole post-ganglionic trunk was included in the ligature embracing the nervous structures cranial to the ganglion, i.e. nerves IX, X, XI, and XII, to prevent leakage of perfusion fluid. The reservoirs containing the perfusion fluids were kept in a water-bath at 38° C. When one fluid was substituted for another, the dead space of the perfusion system was rapidly flushed through a side arm in the inflow cannula in the common carotid artery, and the common carotid artery itself flushed through a second cannula inserted into the lingual artery.

Stimulation. The preganglionic trunk was separated from the vagus and stimulated through bare platinum electrodes with pulses of 0·5 msec duration delivered through a stimulus

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isolation unit. Biphasic pulses were used to avoid polarization (action potential records showed that such stimuli did not set up repetitive responses). A liquid paraffin pool bathed the preparation and the nerve was not moved on the electrodes throughout the experiment. The frequency of stimulation was 2/sec except where otherwise noted. Occasionally continuous stimulation was employed, but since ACh output falls off rapidly with such stimulation (see MacIntosh, 1959) short periods of stimulation were usually alternated with similar periods of rest. This latter procedure, which was previously adopted by Hutter & Kostial (1954), led to the release of approximately equal amounts of ACh in successive periods of stimulation and allowed experimentally induced variations in ACh output to be more easily examined. The usual procedure was to stimulate for 5 min and to collect all the effluent during this period and the next 2 min. In this way a 'stimulation sample' was obtained containing all the ACh released by the stimulus without any being carried over into the next sample as a consequence of dead space in the collecting system. Then followed a period of 5 min during which a 'resting sample' of perfusate was collected in the absence of stimulation. This cycle was repeated as often as necessary.

Solutions. Four different perfusion fluids were used. (1) Locke's solution contained (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·2, Na_2HPO_4 2·15, NaH_2PO_4 0·85, glucose 10·0. (2) Locke's solution with barium was as (1) except that 6 mM $BaCl_2$ was added. (3) Barium-substituted Locke's solution was as (1) except that $CaCl_2$ was replaced by an equimolar amount of $BaCl_2$ (i.e. 2·2 mM). (4) Calcium-free Locke's solution was as (1) except that $CaCl_2$ was omitted. All perfusions contained 10^{-5} g eserine sulphate/ml. and were equilibrated with pure O_2. Their pH was close to 7·0.

Precipitation of barium. Before testing the ACh content of the barium-containing perfusates, barium was precipitated by the addition of Na_2SO_4 in amounts sufficient to leave an excess of sulphate of about 10 mM; the $BaSO_4$ thus precipitated was removed by filtration through a sintered glass filter. Separate measurements showed that ACh added to solutions which were treated in this way could be quantitatively recovered, and that any remaining traces of barium or the excess sulphate did not cause any detectable effect on the cat's blood pressure in the amounts injected during the assays.

Assay. ACh was assayed by its depressor effect on the systemic blood pressure of the eviscerated cat under chloralose (MacIntosh & Perry, 1950). Blood-pressure records were made with a pressure transducer and ink-writing oscillograph. Sample tests showed that the depressor effects were abolished by atropine sulphate 0·5 mg/kg I.V., and that no depressor activity appeared in the samples when eserine sulphate was omitted from the perfusion fluid.

RESULTS

Locke's solution with barium

The addition of 6 mM of barium to Locke's solution caused a striking increase in the amount of ACh liberated by preganglionic stimulation in each of four experiments. All these were performed in the manner illustrated in Fig. 1; thus, perfusion was begun with Locke's solution and four control samples of effluent were collected. The first and third of these were obtained in the absence of nerve stimulation; the second and fourth during preganglionic stimulation at 2 shocks/sec. Immediately after the second period of stimulation (i.e. the fourth sample) perfusion with Locke's solution was suspended and perfusion began with Locke's solution with barium. Again four samples were collected, samples 5 and 7 in the absence of stimulation and 6 and 8 during stimulation. Finally, perfusion was

resumed with Locke's solution and two more resting samples (9 and 11) and two more stimulation samples (10 and 12) were obtained.

In all these experiments the ACh content of the samples collected during stimulation in the presence of barium (samples 6 and 8) exceeded that obtained in Locke's solution alone. In three of the four experiments the effect was much more pronounced in the first of these periods of stimulation in the presence of barium (as in Fig. 1); but in the fourth experiment

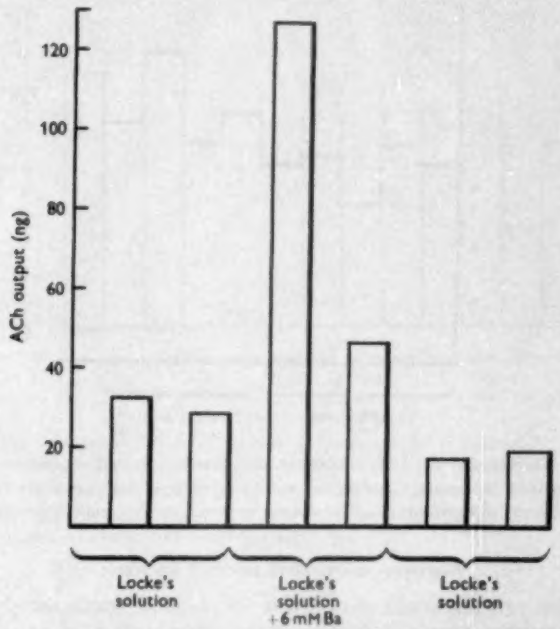

Fig. 1. Increased ACh output caused by adding 6 mM of barium to Locke's solution. Each block represents ACh output from the perfused superior cervical ganglion for a 5 min period of preganglionic stimulation at 2/sec.

the two samples obtained during stimulation in barium were equally active. Barium approximately doubled ACh output in three of these experiments and caused about a fourfold increase in another (Fig. 1).

The samples obtained in the intervals between periods of stimulation in Locke's solution usually contained no ACh detectable by the assay method used. Occasionally, when the assay cat was particularly sensitive, a few nanograms of ACh were detected in these 'resting samples'. No obvious increase in this 'resting output' of ACh occurred during the first period after switching from Locke's solution to Locke's solution with barium

(sample 5) but in one experiment the samples collected following stimulation in the presence of barium contained more ACh than the control resting samples collected in Locke's solution: the amount was too small to be assayed accurately. It was clear, however, from the assays of these 'resting samples' that any effect of barium on ACh output in the absence of stimulation could not account for the large output which accompanied preganglionic stimulation.

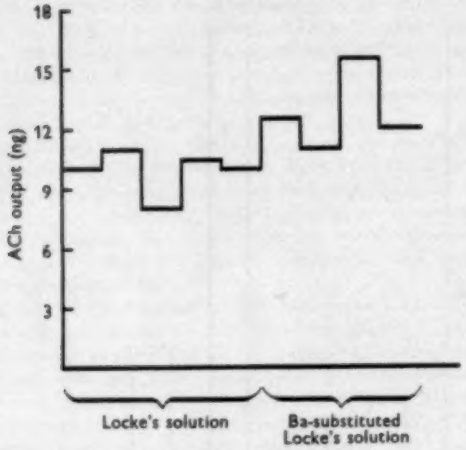

Fig. 2. Maintenance of ACh output on substituting barium for calcium. The values plotted represent the ACh output in successive 5 min periods from the perfused superior cervical ganglion during continuous preganglionic stimulation at 1/sec.

Barium-substituted Locke's solution

When the preganglionic trunk was stimulated continuously at 1/sec while the ganglion was perfused with Locke's solution, the output of ACh was low but well sustained. If the Locke's solution were changed for another, identical in all respects except that calcium was replaced by an equimolar amount of barium (barium-substituted Locke's solution), ACh output was maintained (Fig. 2). Calcium-free solutions do not support ACh output in this way (Harvey & MacIntosh, 1940; and below) and it therefore appeared that barium could be substituted for calcium in these conditions. This possibility was more rigorously tested in experiments in which calcium deprivation (and a fall in ACh output) was established before testing the effect of barium. In such experiments the ganglion was first perfused with Locke's solution. Then this perfusion was changed to calcium-free Locke's solution and ACh output was shown to fall. Thereafter perfusion was continued with barium-substituted Locke's

solution, and finally again with Locke's solution. In each of these experiments ACh output fell to very low values with calcium-free Locke's solution. But soon after beginning with barium-substituted Locke's solution ACh output recovered to levels similar to those found in Locke's solution (Fig. 3). No ACh was detected in the samples obtained during perfusion with barium-substituted Locke's solution in the absence of stimulation.

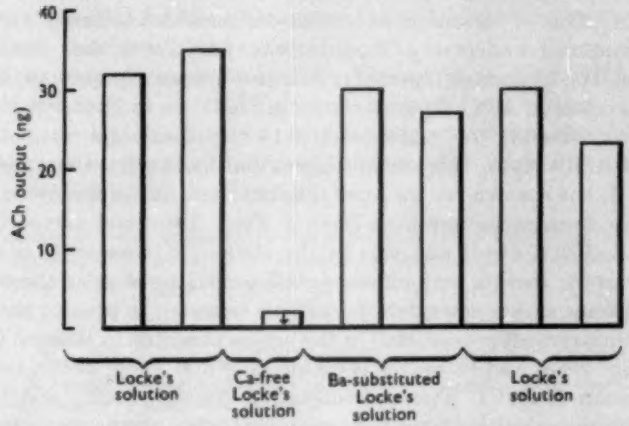

Fig. 3. The restorative effect of barium on ACh output depressed by calcium deprivation. Each block represents ACh output from the perfused superior cervical ganglion for a 5 min period of preganglionic stimulation at 2/sec. Note: no output of ACh in the absence of calcium was detected; the value plotted gives an upper estimate based on the sensitivity of the assay.

DISCUSSION

The effect of barium at the superior cervical ganglion resembles that of calcium, in that when added to Locke's solution it augments ACh output and when added to calcium-free Locke's solution it restores ACh output. Although calcium has an obviously important role in controlling ACh release its mode of action is obscure. Hodgkin & Keynes (1957) have demonstrated that there is an increased entry of calcium ions into squid nerve during activity, and that this increases with increasing extracellular calcium-ion concentration, as does the efflux of ACh from the ganglion. They have also found that this influx of calcium is depressed by magnesium ions, as is the efflux of ACh from the ganglion. Both they, and Birks & MacIntosh (1957) have postulated that calcium ions release ACh by entering the nerve terminals during the impulse and disrupting the ACh-retaining structures. It seems probable that barium acts in the same way. Barium

is not only closely related to calcium in its chemical properties, but also appears to penetrate axons during the impulse; thus Greengard & Straub (1959) have observed action potentials in mammalian B and C fibres maintained in isotonic barium chloride, and have concluded that the inward current is carried by barium ions.

The hypothesis that barium acts in much the same way as calcium on what we might loosely refer to as the 'calcium receptor for ACh release' readily explains the two principal observations in our experiments; the stimulant effect of barium on ACh release when added to Locke's solution, and its restorative effect on ACh output when added to calcium-free Locke's solution. The hypothesis, however, does not adequately account for such large increases in ACh output as shown in Fig. 1, for in Hutter & Kostials' (1954) experiments the addition of 6–14 mM of calcium no more than doubled ACh output. This would suggest that barium has some additional action. It has been known for some time that barium can lead to repetitive responses from nerve terminals (Dun & Feng, 1940) and axons (Lorente de Nó & Feng, 1946) and this might obviously increase ACh output. Alternatively, barium may influence ACh output by altering the shape of preganglionic action potentials, for barium is known to prolong the falling phase (negative after-potential) of the action potential in muscles (Fatt & Ginsborg, 1958) and in nerves (Lorente de Nó & Feng, 1946), including mammalian B and C fibres (Greengard & Straub, 1959); and there is electrophysiological evidence indicating that when the action potential is thus increased (as for example by passing hyperpolarizing currents through the nerve terminals) the output of 'transmitter' is increased (del Castillo & Katz, 1956).

Whatever the explanation, it is clear that barium increases the amount of ACh released by preganglionic stimulation, and it is of interest to relate this to some of its known pharmacological actions. Although the best known action of barium is its direct spasmogenic effect on smooth muscle, Ambache (1946) and Feldberg (1951) obtained evidence that it could also excite ganglion cells in the intestine; and Ambache (1949) showed that barium excited ganglion cells when injected into the superior cervical ganglion. These effects of barium in the intestine or superior cervical ganglion were depressed by hexamethonium or nicotine. It is possible that some of the ganglion-stimulating effect of barium is due to its increasing ACh release from active presynaptic terminals. This would certainly account for the inhibitory effects of nicotine and hexamethonium. It must be mentioned, however, that Ambache (1949) was able to obtain stimulant effects of barium on the superior cervical ganglion after chronic degeneration of preganglionic endings, so that barium seems to be able to stimulate ganglion cells directly. Barium is also known to have an

eserine-like effect at the neuromuscular junction (Feng, 1937), and it will be of interest to see whether this too is attributable to barium increasing ACh output.

SUMMARY

1. Experiments have been made on the effect of barium on the release of ACh from the perfused superior cervical ganglion of the cat.

2. The addition of 6 mM of barium to the Locke's solution used to perfuse the ganglion increased the amount of ACh released by preganglionic stimulation about twofold or more.

3. When calcium was absent from the perfusion fluid, ACh output could be sustained or restored by the addition of 2 mM barium.

4. It is suggested that barium may act in part by participating in the calcium-receptive mechanisms for ACh release, and in part by altering the action potentials of preganglionic nerves.

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REFERENCES

AMBACHE, N. (1946). Interaction of drugs and the effect of cooling on the isolated mammalian intestine. *J. Physiol.* **104**, 266–287.

AMBACHE, N. (1949). The nicotinic action of substances supposed to be purely smooth-muscle stimulating. (B) Effect of $BaCl_2$ and pilocarpine on the superior cervical ganglion. *J. Physiol.* **110**, 164–172.

BIRKS, R. I. & MACINTOSH, F. C. (1957). Acetylcholine metabolism at nerve endings. *Brit. Med. Bull.* **13**, 157–161.

DEL CASTILLO, J. & KATZ, B. (1956). Biophysical aspects of neuro-muscular transmission. *Progr. Biophys.* **6**, 122–170.

DOUGLAS, W. W., LYWOOD, D. W. & STRAUB, R. W. (1960). The effect of barium on the release of acetylcholine from the cat's superior cervical ganglion. *J. Physiol.* **154**, 39–40 *P*.

DUN, F. T. & FENG, T. P. (1940). Studies on the neuro-muscular junction. XX. The site of origin of the junctional after discharge in muscles treated with guanidine, barium or eserine. *Chin. J. Physiol.* **15**, 433–444.

FATT, P. & GINSBORG, B. L. (1958). The ionic requirements for production of action potentials in crustacean muscle fibres. *J. Physiol.* **142**, 516–543.

FELDBERG, W. (1951). Effects of ganglion blocking substances on the small intestine. *J. Physiol.* **112**, 177–196.

FENG, T. P. (1937). Studies on the neuromuscular junction. VII. The eserine-like effects of barium on motor nerve endings. *Chin. J. Physiol.* **12**, 177–195.

GREENGARD, P. & STRAUB, R. W. (1959). Restoration by barium of action potentials in sodium-deprived mammalian B and C fibres. *J. Physiol.* **145**, 562–569.

HARVEY, A. M. & MACINTOSH, F. C. (1940). Calcium and synaptic transmission in a sympathetic ganglion. *J. Physiol.* **97**, 408–416.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HUTTER, O. F. & KOSTIAL, K. (1954). Effect of magnesium and calcium ions on the release of acetylcholine. *J. Physiol.* **124**, 234–241.

KIBJAKOW, A. W. (1933). Über humorale Übertragung der Erregung von einem Neuron auf das Andere. *Pflüg. Arch. ges. Physiol.* **232**, 432–443.

LORENTE DE NÓ, R. & FENG, T. P. (1946). Analysis of effect of barium upon nerve with particular reference to rhythmic activity. *J. cell. comp. Physiol.* **28**, 397–464.

MACINTOSH, F. C. (1959). Formation, storage and release of acetylcholine at nerve endings. *Canad. J. Biochem. Physiol.* **37**, 343–356.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine. *Methods med. Res.* **3**, 78–92.

PERRY, W. L. M. (1953). Acetylcholine release in the cat's superior cervical ganglion. *J. Physiol.* **119**, 439–454.

J. Physiol. (1961), **156**, *pp.* 523–543
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THE PREPARATION AND PROPERTIES OF GASTRIN

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J. S. Edkins (1905, 1906) was the first to report that extracts of pyloric mucosa stimulated gastric secretion when injected intravenously into anaesthetized cats. He suggested that the active principle, to which he gave the name 'gastrin', exercised a hormonal role in the stimulation of gastric secretion following a meal. At the time there existed little or no evidence from physiological experiments to support such an hypothesis; and although Edkins claimed that in the cat and dog gastrin activity was largely confined to the pyloric region, it was soon shown by those who attempted to confirm his results that simple extracts made from all regions of the stomach, and indeed from most tissues in the body, contained a strong stimulant of gastric secretion (Lim, 1922). This ubiquitous secretagogue was eventually identified as histamine (Barger & Dale, 1910; Dale & Laidlaw, 1910; Popielski, 1919), and the failure of attempts to find in pyloric mucosal extracts any stimulant of gastric secretion other than histamine (Sacks, Ivy, Burgess & Vandolah, 1932; Gavin, McHenry & Wilson, 1933) fostered a general belief that the activity of Edkins's extracts was most probably attributable to the presence of that substance.

This view was radically changed by the work of Komarov (1938, 1942*a*, *b*) who recognized that the antral hormone might be of protein nature. If so, it would have been removed, but histamine spared, in the deproteinizaton procedures employed by all those who had previously sought to identify the secretagogue present in their extracts. Komarov showed that trichloroacetic acid would precipitate from an acid aqueous extract of antral mucosa a protein fraction which was apparently free from histamine but which on injection into anaesthetized cats and conscious dogs would stimulate the secretion of gastric acid, though not of pepsin. In the stomach the active principle was found only in the pyloric region; a small amount was detected in duodenal extracts. The preparations described by Komarov were of low potency, though non-toxic when injected intravenously, and did not stimulate secretion when injected subcutaneously into conscious dogs provided with gastric fistulae or pouches. This was regarded as further evidence that the active principle was of protein nature and therefore not readily absorbed when administered

in this way, and also that the power of the extracts to excite secretion when injected intravenously or intramuscularly was not due to the presence in them of histamine.

Methods for the preparation of gastrin were later described by Uvnäs (1943*a*, *b*, 1945*a*), Munch-Petersen, Rönnow & Uvnäs (1944), Harper (1946) and Jorpes, Jalling & Mutt (1952). These workers all tested their extracts by intravenous injection into anaesthetized cats, and expressed the potencies of them in similar terms to that introduced by Komarov (1942*a*); one unit of gastrin caused the secretion of 1 ml. of acid gastric juice in an anaesthetized cat during 1 hr after an intravenous injection. Komarov's most potent preparations contained one unit in 2·5 mg; Uvnäs (1945*a*) and Jorpes *et al.* (1952) obtained preparations having a potency of 1–10 units/mg.

Although these investigations establish beyond doubt that gastrin extracts made by the methods described will stimulate secretion in anaesthetized cats, very little evidence has been brought to show that such preparations are active when injected into conscious animals; only two descriptions of such an experiment exist. Komarov (1942*b*) gave 200 mg of one of his preparations ('purified gastrin I') as a single intravenous dose to a conscious gastric-fistula dog; it caused the secretion during the next 70 min of 20·5 ml. of acid gastric juice. The same amount of the same extract, injected intravenously in five divided doses into an anaesthetized cat, caused the secretion of 8·6 ml. of juice. Linde (1950) injected slowly intravenously (25 mg/hr) into conscious dogs provided with gastric pouches a preparation made by Jorpes & Mutt, which had been shown to be highly active in the anaesthetized cat. In two of Linde's experiments there was no response; in the other two a small secretion occurred, the maximal rate being 1·7 ml. in 10 min. Ivy (1941) and Grossman (1950) reported that their efforts to prepare gastrin extracts were usually unsuccessful, and there is reason to believe that they tested their preparations by injection into conscious gastric-pouch or fistula dogs.

A gastrin preparation which would stimulate gastric secretion in conscious animals as effectively as does histamine would clearly be of the greatest value for many purposes, including clinical studies. In fact, the work with which this paper is concerned arose out of the need to obtain a gastrin extract which would stimulate secretion in the conscious dog, and against which could be tested the action of fat in the duodenum, with a view to elucidating the mechanism by which the hormone enterogastrone inhibits gastrin secretion (Gregory & Tracy, 1959*a*). The method which has been evolved provides a highly purified and potent preparation which stimulates the secretion of gastric acid but not of pepsin in conscious gastric-fistula and pouch dogs. In contrast with all preparations previously

described, it is effective on subcutaneous injection, as well as intravenously or intramuscularly; and it has been shown to be suitable for experiments in the human subject. A preliminary account of the work has already been given to the Physiological Society (Gregory & Tracy, 1959*b*, 1960).

Development of the method

At the outset attempts were made to repeat the methods of preparation described by previous investigators, testing the products by slow intravenous injection into conscious gastric-pouch and fistula dogs. The experience thus gained showed that two major problems had to be solved before a gastrin preparation could be obtained which might be suitable for refinement by such techniques as chromatography or electrophoresis. The first of these was to find a satisfactory method of initial extraction of the fresh tissue. Fresh antral mucosa is very tough and cannot easily be ground or minced; furthermore, aqueous solvents such as dilute acid extract a large amount of mucin, which interferes with subsequent deproteinization. Komarov, and Uvnäs and his co-workers, extracted the fresh mucosa with hot dilute acid; some inert protein was removed by partial neutralization and the supernatant fluid then treated with trichloroacetic acid. In our hands this method proved unsatisfactory since the initial acid extraction resulted in a broth-like fluid, and when this was treated with trichloroacetic acid, there was obtained only a very small precipitate which contained but little activity.

Harper (1946) used the ethanol extraction introduced by Mellanby (1932) for the preparation of secretin, but the procedure was not fully described in the preliminary account of the work and no further report was made. Jorpes *et al.* (1952) extracted the mucosa (which was previously heated to stop enzyme action) with acid methanol, and collected the precipitate which appeared when this was adjusted to pH 7. This method appeared to offer great promise, but trials of it were disappointing; the activity of the product was found to be very low when tested as described above.

However, these instances of the use of non-aqueous solvents suggested a procedure based on that used by Lim, Ling & Liu (1934) in their attempts to extract enterogastrone from hog intestine. They treated the fresh duodenal mucosa with picric acid, which precipitates protein and stops enzyme action, and then extracted it with 80% acid ethanol. The active material was recovered by the addition of a large volume of acetone. This procedure was modified for the extraction of antral mucosa, acetone being used instead of ethanol, and the extract being treated with ether to remove picric acid and acetone, so that there finally remained a relatively small aqueous residue. This, when freed from ether, could be cleanly precipitated by trichloroacetic acid. The heavy protein precipitate thus obtained was

soluble in dilute acid, and it proved to be rich in gastrin activity. This method of obtaining a crude aqueous extract of the mucosa was used until comparatively recently, and reference is made to it in the preliminary accounts of the work reported here. It also formed the basis of the extraction procedure used by Gregory, Tracy, French & Sircus (1960) in their demonstration of the presence of gastrin-like activity in the pancreatic tumour from a patient showing the Zollinger–Ellison syndrome. However, further studies led to the later introduction of a new and superior procedure for the initial extraction of the fresh mucosa, based upon the observation that gastrin is highly soluble in aqueous acetone in the presence of trichloroacetic acid. The antrums obtained at the abattoir were immersed in a 4% solution of trichloroacetic acid after inversion and trimming. In the laboratory the mucosa was removed and extracted during the night with acetone containing 4% trichloroacetic acid; the quantity of acetone used in relation to the weight of the mucosa was such that the final concentration of acetone in the extract was approximately 70–80%. The extract was freed from trichloroacetic acid and acetone by the addition of HCl and extraction with ether.

The crude aqueous residue obtained by either of these methods of extraction contained large amounts of denatured protein which was insoluble between pH 4 and 10, and when precipitated adsorbed the greater part of the gastrin activity. The bulk of this inert protein could be removed by precipitating it in hot solution at pH 8·5 in the presence of approximately 2% NaCl; the clear supernatant fluid was rich in gastrin. The latter could be completely recovered, together with some remaining protein which acted as a 'carrier', by saturation with sodium chloride at room temperature at pH 4·5. The precipitate which formed was easily collected by filtration and then dissolved in a relatively small volume of 0·1 N-HCl. This solution was freed from more inert protein by repetition of the precipitation in hot solution at pH 8·5. The supernatant from this procedure was then precipitated twice with trichloroacetic acid, which removed NaCl and remaining traces of histamine. The second precipitate was converted by treatment with acid acetone and ether into a powder which was freely soluble in water, the solution having a pH of about 3. On neutralization there appeared a precipitate which contained most of the gastrin activity; it redissolved at pH 8–9. Extracts made from this powder were free from significant amounts of histamine and showed excellent evidence of gastric activity when injected into conscious dogs in amounts equivalent to 10–20 g of mucosa.

The next step was to find some means of freeing the gastrin activity from the residuum of denatured protein which still formed the bulk of the crude powder described above, and this proved to be the second major

problem in the purification of the hormone. A variety of protein precipitation procedures were tried without success; the only one which offered any promise was that introduced by Ivy & Greengard (1938) in their preparation of crystalline secretin, in which inert protein was precipitated from an acid solution in 80 % acetone by the addition of aniline. When this procedure was applied to the crude powder described above, recovery of gastrin activity in the supernatant was found to be variable, and in general inadequate; but the results prompted a study of the influence of pH on the precipitation of inert protein from a solution of the powder in acid aqueous acetone. It was eventually found that by dissolving the powder in water at pH 10 by addition of a strong organic base (piperidine), adding acetone to 80 % and then reducing the pH to 8·5 by addition of an organic acid (acetic) most of the inert protein present (about 80 %) could be cleanly precipitated, while the gastrin activity remained in the clear supernatant fluid. From this it could be recovered in the aqueous residue resulting from ether extraction.

The solution thus obtained had a pH of approximately 7, and contained about 0·2 % NaCl. It was found to be suitable for fractionation on a column of calcium phosphate gel, the use of which in this connexion had already been contemplated in view of its successful use in the purification of urogastrone (Gregory, 1955). At an appropriate pH the gastrin present in the solution was completely retained by such a column, and after washing with water, which removed almost all the biuret-reacting material present, the gastrin could be completely eluted by a low concentration of phosphate. The actual weight of material thus obtained could not be determined, since no satisfactory means could be found of freeing the solution from phosphate, but microchemical analysis showed that an amount of the product equivalent to 1 kg of mucosa contained about 0·5 mg total N. These observations were combined to form the method now to be described.

METHODS

Stage 1. Hog antrums are obtained at the abattoir as soon as practicable after killing. They are turned inside out, washed briefly in cold water and trimmed free of duodenal and corpus tissue. They are then immersed in 4–5 % trichloroacetic acid solution and brought to the laboratory. Twenty-four to thirty antrums provide about 1 kg of mucosa, and the ensuing directions refer to the processing of this amount of material.

Each antrum is slit open and the mucosa removed in large strips with toothed forceps and curved scissors. The strips are weighed and then immersed in a volume of acetone (containing 4–5 % trichloroacetic acid) equivalent to twice their weight. The vessel used should be shallow, so that the strips occupy most of the volume of liquid, and must be well covered. The contents are stirred from time to time and then left during the night at room temperature. The next morning the mixture is stirred up and then decanted. It is filtered through coarse fluted papers (Green 904½) into two 4-l. bottles; the filtrate is dark and usually cloudy. To each bottle is added 5 ml. of 10 N-HCl and two volumes of ether. The

mixture is well shaken for 1–2 min and then left until separation is complete, when the clear brown ethereal phase is removed by siphon or suction. The two aqueous portions are combined in a small glass-stoppered bottle and extraction continued as before with three successive portions of ether (1 volume). The volume of the aqueous residue is measured and its pH adjusted to 3 with 2 N-NaOH.

It is cautiously heated in a large evaporation dish on an electric water-bath, preferably in a current of air, until all the ether is removed, and then transferred to a beaker; heating is continued to 70–80° C. The pH is then raised first to 5–5·5 by slow addition of 2 N-NaOH, with constant stirring, and after 5–10 min at the same temperature to 8·5 by addition of ammonia solution (0·880 ammonia diluted with an equal volume of water); the temperature is then maintained for a further 5 min. The mixture is briefly centrifuged and the clear golden-yellow supernatant fluid collected; the pH is reduced to 4·5 by addition of 10 N-HCl.

The residue in the centrifuge cups is suspended in a volume of 0·1 N-HCl equal to that of the original aqueous residue and heated to 70–80° C for 5–10 min; partial resolution occurs. One-twentieth volume of 30 % NaCl solution is added; the pH is raised to 5–5·5 by addition of 2 N-NaOH, and after 5–10 min to 8·5 by addition of ammonia solution. The temperature is maintained for 5 min and the mixture then briefly centrifuged. The clear faintly pigmented supernatant is brought to pH 4·5 and added to the first, and the solution cooled to room temperature.

Sodium chloride crystals, to give a final concentration of 30 %, are now added with constant stirring by a motor-driven propeller, and after 60 min the flocculent precipitate is collected. This may be done by centrifuging at high speed for 15–20 min but a more convenient and effective method is by filtration, using a glass-fibre paper (Whatman) supported on a coarse paper (Green 904) and adding 2 g of acid-washed 'Hyflo Supercel' to the suspension. With moderate suction filtration is rapid; the cake is washed with 30 % NaCl solution before being sucked as dry as possible. The cake is then suspended in 100 ml. of 0·1 N-HCl. The suspension is heated to 70–80° C; after 5–10 min the pH is raised first to 5–5·5 with 2 N-NaOH and then after a further 5 min to 8·5 with ammonia solution. The temperature is maintained for 5 min and the suspension then briefly centrifuged. The clear pigmented supernatant is collected and its pH brought to 7 by addition of 10 N-HCl.

The residue in the centrifuge cups is suspended in 100 ml. of 0·1 N-HCl and the temperature of the suspension raised to 70–80° C. After 5–10 min, sodium chloride (4·5 ml. of 30 % solution) is added and the procedure of precipitation at pH 5–5·5 and 8·5 repeated as before. The second supernatant is brought to pH 7 and combined with the first.

The volume of the solution is measured and it is cooled to 10° C. An amount of trichloroacetic acid crystals calculated to give a final concentration of 4–5 % is dissolved in a small volume of water (20 ml.) and added to the cooled supernatant at a slow drip rate with constant stirring by a motor-driven propeller; the addition should take at least 15 min. The mixture is left in the refrigerator for a further 45 min and then centrifuged for 5 min. The perfectly clear supernatant is poured off and the insides of the cups wiped dry. The precipitate is stirred up in water to a total volume equal to that of the original combined supernatants; it dissolves, forming a clear green solution, and gentle warming will facilitate the process. It is important to disperse all lumps while the volume in which the precipitate is suspended is still small. When solution is complete, the liquid is cooled to 10° C and precipitated with trichloroacetic acid as before.

After centrifuging for 10 min and draining and wiping out the cups, the precipitate in each is stirred up in a very small volume of acetone (2–5 ml.). The precipitate can usually be made to dissolve to a green viscid opalescent solution, due to the presence in it of residual aqueous trichloroacetic acid, but if this cannot be accomplished, all lumps are dispersed with a spatula as finely as possible before adding acetone with constant stirring so as to half-fill the cup. A heavy flocculent precipitate with curdy supernatant forms. Hydrochloric acid (10 N) is now added, with stirring, in the proportion of 2 ml. to 100 ml. acetone,

and the mixture well shaken; any remaining lumps must be finely dispersed with a spatula. Finally, ether is added to fill the cup and the mixture well shaken. After a few minutes the cups are centrifuged for 5 min at high speed. The clear supernatant is poured off and the gummy precipitate resuspended finely in acetone. After centrifuging, the acetone is removed by suction; this must be done with care, as the precipitate is now granular and does not pack well. The precipitate is finally washed in the same way with ether, which is also removed by suction. The cups are left on their sides for a few hours or during the night; the precipitate dries out perfectly. It is weighed and stored in a bottle in the dark. The yield is 600–700 mg from 1 kg of mucosa; the activity remains unchanged for at least six weeks.

Stage II. The powder obtained from Stage I (approx. 600 mg) is dissolved in 30 ml. of water in a 300 ml. beaker. Solution may be facilitated by warming, but the solution must be cooled to room temperature before proceeding. The pH is first brought to 5–5·5 by addition of 2 N-NaOH; a heavy flocculent precipitate appears. The pH is then raised to 10–10·5 by addition of a few drops of piperidine; the 'purified' reagent (British Drug Houses Ltd.), which is colourless, must be used. The precipitate dissolves completely and a pale green perfectly clear solution results. Acetone (120 ml.) is slowly added, with constant stirring; a curdy precipitate, with cloudy supernatant, forms. Acetic acid (5 ml. of glacial acetic acid diluted to 100 ml. with water) is next added drop by drop, with constant stirring, so as to bring the pH gradually down to 8·5. The mixture is poured into a dry centrifuge cup and vigorously shaken. After standing a few minutes, it is briefly centrifuged and the perfectly clear, almost colourless, supernatant poured off into a one-litre separating funnel. The precipitate is resuspended in a total of 30 ml. of water in the centrifuge cup and a drop or two of piperidine added to effect perfect solution as before. The liquid is then transferred to the original beaker and the precipitate adhering to the sides of this also brought into solution. Sodium chloride (0·1 ml. of a 30% solution) is added, and the precipitation at pH 8·5 in 80% acetone repeated exactly as before; the addition of a small amount of NaCl is necessary at this point, as the second supernatant is otherwise opalescent. The second supernatant is poured into a second separating funnel and about 600 ml. of ether added to each. The contents are well shaken and the aqueous phases removed and combined after separation is complete. The ethereal phases in each funnel are shaken with 20 ml. portions of water, and these are added to the previous aqueous fractions after separation.

The clear almost colourless solution thus obtained is extracted with two to three volumes of ether twice more in a smaller separating funnel or stoppered measuring cylinder (removing the ether by suction) and then transferred to a flask fitted with a side arm and a capillary air inlet which does not dip into the liquid. The flask is evacuated and the liquid swirled about the walls until all ether has been removed; brief warming in water at 30–40° C is permissible.

The faintly pigmented clear solution which finally results has a volume of about 100 ml., contains about 0·2% NaCl, and has a pH approximately 7; it is usually clear but may become faintly cloudy on standing. To ensure complete and permanent solution of the small amount of denatured protein which is still present, the pH is first raised to 9–9·5 with ammonia solution, and then returned to 7·0 with dilute acetic acid. The solution is now ready for Stage III of the preparation. It must be emphasized that Stage II preparations which are definitely cloudy are not suitable for use in Stage III; the denatured protein which has been precipitated adsorbs the greater part of the gastrin present, and this cannot be recovered from the calcium phosphate column.

Stage III. A preparation of calcium phosphate gel suitable for the further purification of the Stage II product is made as follows. Five hundred millilitres of 0·5 molar $CaCl_2$ solution is added to 1 l. of 0·5 molar Na_2HPO_4 solution at a moderate drip rate (about 2 drops/sec) with constant vigorous stirring, using a motor-driven glass propeller. The precipitate which forms is washed repeatedly by decantation (six times is adequate) with

1-l. portions of distilled water until no more than a trace of Cl^- is detectable in the supernatant. The gel is finally made up to a volume of 250 ml. with distilled water and stored in a plastic bottle in the refrigerator; it is not advisable to use it for longer than one month after preparation.

For the routine treatment of Stage II solutions a short column is made from the gel in a simple chromatography tube. This is constructed from a length of glass tubing about 15 cm long and 15–20 mm internal diameter, with a narrow outlet tube about 20 mm long, the entrance to which from the wider tube is blocked by a plug of cotton-wool during use. A short length of rubber tubing, fitted with a screw clip and a glass tip, completes the assembly. There is also required a mixer, consisting of a stainless-steel or Perspex plate machined to fit the tube closely and freely perforated, to which is attached a central metal rod. The outlet of the tube is closed, and about 25 ml. of gel suspension poured in. It is mixed for a moment or two with the implement described above; the fit of this in the tube must not be so close that packing of the gel occurs. The outlet of the tube is then opened and gentle air pressure applied to the top of the column. As liquid leaves the outlet at a moderate drip rate, the column is seen to form perfectly evenly from below upwards; an amount of gel should be used which gives a column 3–4 cm long. When formation of the column is complete, the pressure is released and the outlet closed; some clear liquid remains above the top of the column.

A circle of wet filter paper (Whatman No. 4) cut to fit the end of the mixer is now applied to the latter and brought down to the top of the column; it is important not to trap an air bubble beneath the paper, and the column must not be compressed. The liquid above the column is now removed by suction and it is ready for use.

The Stage II solution is run on without disturbing the column and allowed to pass through at a slow rate (25 ml./hr). It is followed by 50 ml. or more of distilled water at a slightly faster rate. Finally, the activity is displaced by passing a solution of Na_2HPO_4 (M/50, anhydrous salt 0·28 g/100 ml.) at a slower rate (10 ml./hr).

There is no visible guide, either in ordinary or U-V light, to the progress of displacement, and the simplest means of ensuring complete recovery of the activity is to collect a total of 20 ml. of effluent. This contains only a small amount of phosphate, owing to its adsorption on the column. Alternatively, the effluent may be collected in 1–2 ml. fractions and the presence of biuret-reacting material in these determined by the micro-method of Lowry, Rosebrough, Farr & Randall (1951). If this is done it is found that a sharp peak emerges first at the front of the displacing phosphate solution, followed by a 'tail'. By pooling and testing appropriate groups of samples it will be found that the region of the 'peak' contains all the gastrin activity. If the column has been adequately washed before passing the phosphate solution, as indicated by negligible biuret values for the fractions in advance of the 'peak', the active fractions obtained have a very low total N content (approximately 0·5 mg corresponding to 1 kg mucosa). The solution of gastrin thus obtained is colourless and has a pH of approximately 7·0. For use in physiological experiments it is made approximately isotonic by addition of 30 % NaCl solution and stored in plastic bottles in the deep-freeze; the activity remains undiminished for months.

Testing of extracts. The object of the work reported here was to obtain a gastrin preparation which would stimulate secretion in the conscious dog. For this reason no tests were made on anaesthetized animals when the method was being developed. The discovery early in the work that the extracts obtained, though free from significant amounts of histamine, would stimulate secretion when injected subcutaneously in conscious dogs greatly facilitated the work, and this method of injection was subsequently used routinely. No attempt was made at formal assays of active extracts, e.g. by comparison with the response to a standard dose of histamine. Attention was simply directed towards selecting those fractionation procedures which gave the most active preparations when these were injected by the same route (subcutaneously) into the same dogs, in amounts equivalent to the same weight of

mucosa (e.g. 20 g). The response of a given dog to the same preparation on different occasions was found to be consistent, provided that the animal was in a basal state before use (having fasted 12–18 hr). Routine testing was carried out on dogs provided with denervated fundic pouches (Gregory, 1958) or simple gastric fistulae (Gregory, 1955). The latter are much more sensitive, partly because the entire stomach is being used for collection of juice, and partly because the vagal innervation is intact; but it was found important to take account of the liability of some of such animals to secrete spontaneously at the time of an expected meal. A further consideration in the selection of animals for use in testing is the fact that different dogs, whether provided with a denervated fundic pouch or a gastric fistula, vary considerably in their sensitivity not only to gastrin but also to meals and histamine injections. Only those dogs were used which were known to respond well to the latter forms of stimulation, and these animals gave consistently good responses to gastrin injections.

Until some experience of using the method has been gained, it is advisable to test for the presence of activity in the powder obtained at Stage I, and this may be done as follows. An amount of powder equivalent to 100 g mucosa (approx. 60 mg) is heated with 10 ml. of 0·9 % NaCl solution to about 70° on a water-bath until the solution is perfectly clear. The pH is raised to 5–5·5 by dropwise addition of 0·1 N-NaOH, and after 5–10 min at the same temperature, to 8·5 with dilute ammonia solution. The temperature is maintained for a further 5 min and the mixture then cooled to room temperature. It is briefly centrifuged and the perfectly clear faintly pigmented supernatant fluid collected. An amount equivalent to 10–20 g mucosa is taken for injection into a conscious dog. The Stage II solution may be tested after addition of 30 % NaCl solution to approximate isotonicity.

It must, however, be emphasized that if these solutions are opalescent or cloudy when injected the response obtained will be slow in onset, of long duration and relatively small peak magnitude. This is apparently attributable to adsorption of gastrin on imperfectly dissolved denatured protein. However, even when such solutions are perfectly clear, the response obtained is often smaller than that observed on injection of the final preparation. The reason for this is obscure, but it indicates that final judgement as to the total activity present in a preparation should be reserved until the Stage III product has been tested.

RESULTS

Secretory responses in conscious dogs

Subcutaneous injection. Perhaps the most striking observation reported here, and certainly in view of all previous information the most unexpected, is that the preparation described is at all stages effective in stimulating secretion when injected subcutaneously, as well as intramuscularly or intravenously. Figure 1 shows the response of a gastric-fistula dog to a subcutaneous injection equivalent to 20 g mucosa. Secretion began within 15 min of injection and reached its peak after about 60 min. The maximal rate of acid output was about half that obtainable in the same dog with histamine. The response of the same animal to the subcutaneous injection of 1 mg histamine acid phosphate is shown for comparison.

The responses of dogs provided with completely denervated fundic pouches to subcutaneous injections of the preparation were closely similar, except that secretion did not commence within 15 min after injection unless the dose was a large one, and the response was often quite protracted lasting 2–3 hr before coming to an end (Fig. 2). In neither type of pre-

paration was any evidence obtained of a stimulation of pepsin secretion. In gastric-fistula dogs the pepsin concentration in the juice, measured by the method of Hunt (1948), fell to a low level as the response became established, and the calculated output values remained substantially unchanged throughout the response. In fundic-pouch dogs the pepsin concentration fell to values not significantly greater than the controls.

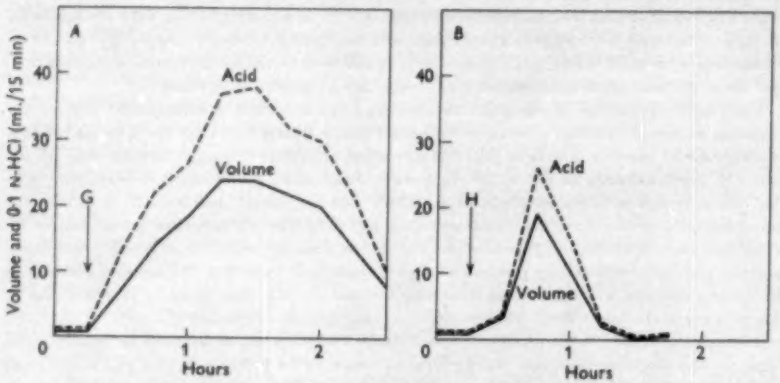

Fig. 1. Response of a conscious gastric fistula dog (weight 10 kg) to a subcutaneous injection (at G) of gastrin (equivalent to 20 g mucosa). At H (another experiment) a subcutaneous injection of 1 mg histamine acid phosphate.

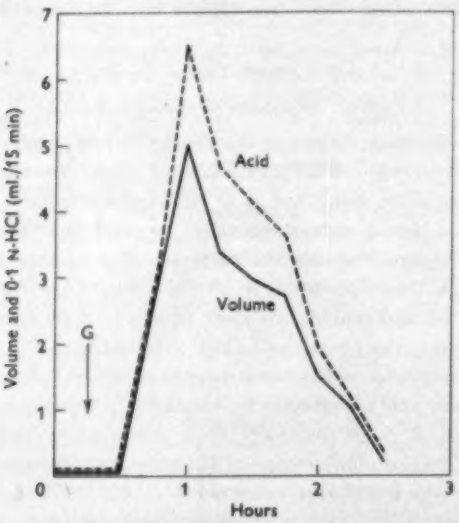

Fig. 2. Response of a denervated fundic pouch in a conscious dog (weight 19·5 kg) to a subcutaneous injection at G of gastrin (equivalent to 20 g mucosa).

Intramuscular injection. The responses of dogs provided with either a gastric fistula or a denervated fundic pouch to intramuscular injections were closely similar in form to those obtained on subcutaneous injection, though sometimes somewhat smaller in total magnitude.

Intravenous injection. Single intravenous injections were relatively ineffective in exciting secretion. In dogs with denervated fundic pouches the intravenous injection of an amount of the preparation equivalent to 20 g mucosa was not usually followed by any significant secretory response. In gastric-fistula animals, which are much more sensitive, a response was

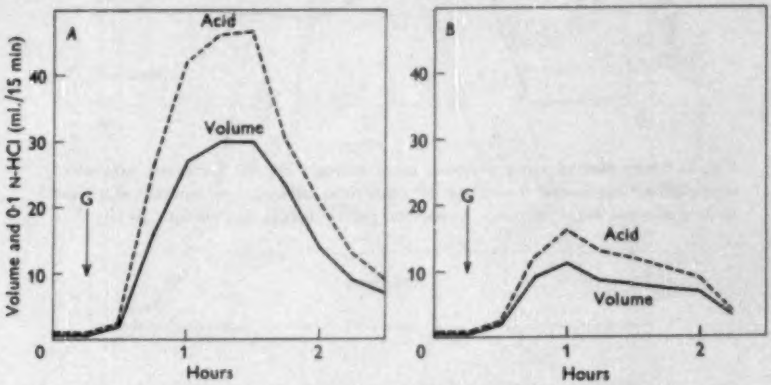

Fig. 3. Responses of a conscious gastric fistula dog (weight 10 kg) to (*A*) subcutaneous and (*B*) intravenous injection of gastrin (G) equivalent to 30 g mucosa.

sometimes obtained, but it was small compared with that produced by a similar injection given subcutaneously (Fig. 3). Continuous intravenous injection of a dose, e.g. equivalent to 20 g mucosa, over a considerable period of time (30–60 min), or the repeated injection of small fractions of the total dose at frequent intervals (5 min) caused responses which compared closely with those obtained when the same amount of material was given as a single subcutaneous injection.

The effect of atropine. When the preparation was injected subcutaneously at regular intervals (30 min), or given continuously intravenously by a motor-driven syringe, a steady secretory response could be maintained for hours without significant decline, even at rates which approximated to the maximal obtainable by the similar administration of histamine. If during such a steady near-maximal response in a gastric-fistula dog atropine was injected intravenously, there occurred a considerable and sustained reduction in the rate of secretion (Fig. 4). However, a comparable or smaller response maintained in the same dog by the similar injection of histamine was almost unaffected by the same dose of atropine.

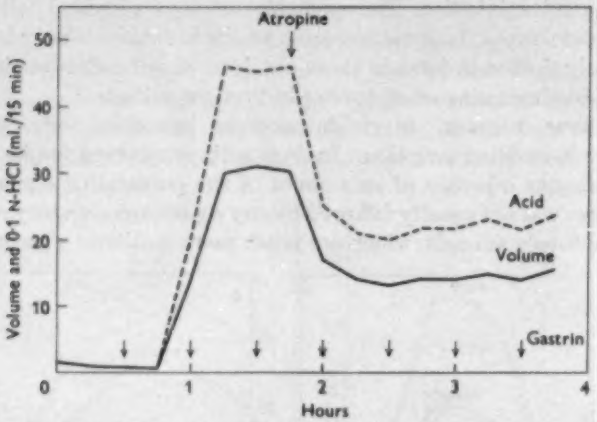

Fig. 4. The effect of 1 mg atropine intravenously on the secretory response to repeated subcutaneous injections of gastrin at arrows (an amount equivalent to 10 g mucosa every 30 min). Conscious gastric-fistula dog (weight 10 kg).

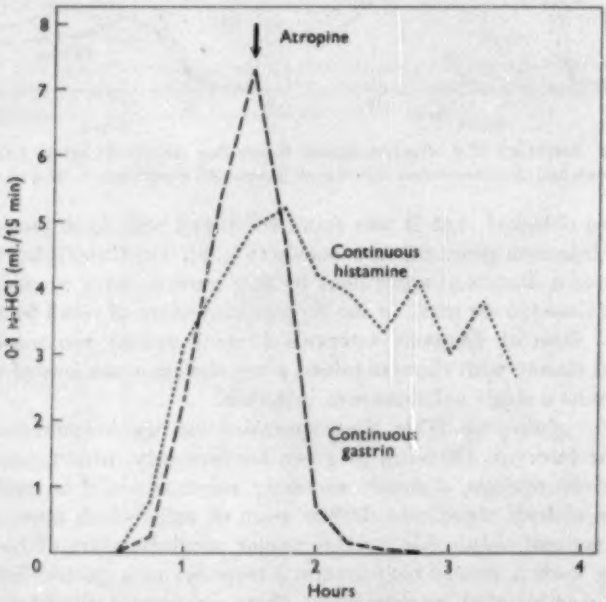

Fig. 5. The effect of 1·2 mg atropine intravenously (at arrow) on the secretory responses of a denervated fundic pouch in a 25·5 kg dog to continuous stimulation with (*a*) gastrin (an amount equivalent to 10 g subcutaneously every 30 min), and (*b*) histamine (0·1 mg acid phosphate subcutaneously every 15 min).

In dogs provided with denervated fundic pouches the depression of a gastrin response by atropine usually amounted to almost complete suppression, whereas in the same animal a similar response to histamine was hardly affected (Fig. 5).

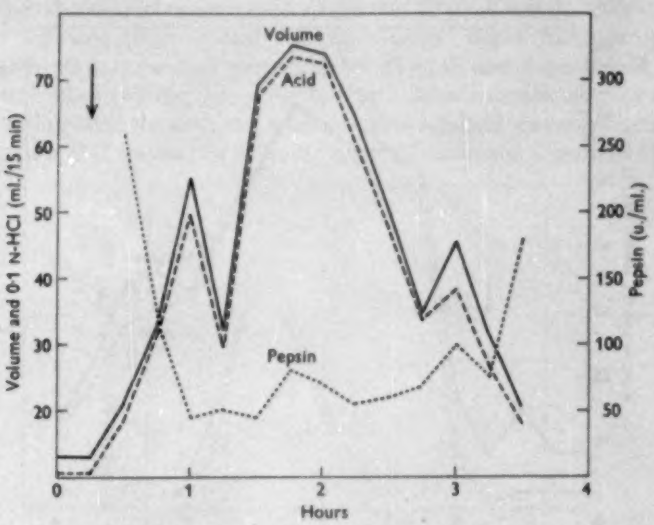

Fig. 6. Response of the human subject to a subcutaneous injection of gastrin equivalent to 200 g mucosa.

Secretory responses of a human subject

In eight experiments the preparation was injected into a normal human subject, and the gastric secretory responses determined by withdrawal of gastric juice at 15-min intervals, using intermittent manual suction; care was taken to prevent contamination by swallowed saliva. The amount injected was equivalent to 200 g of fresh mucosa and contained approximately 0·1 mg total N; it was dissolved in approximately 1 ml. 0·9 % NaCl solution. The material used was derived from four different batches of gastrin.

In four experiments the injection was subcutaneous, in two intramuscular and in two intravenous. The experiments were performed at intervals of one week or more. The subject experienced no significant side or after effects, except in the case of the intravenous injections. The first of these was given rapidly, i.e. during 15–30 sec; this caused a definite but transient sinking feeling in the chest and abdomen, which passed off in less than 1 min. There was no change in heart rate or respiration, and no flushing or sensation of warmth. The second intravenous injection,

from another batch of material, was given on another occasion over a period of 5 min; the subject detected a very slight sensation similar to that just described. In the other experiments no effects whatever were noticed. The subcutaneous and intramuscular injections caused no more

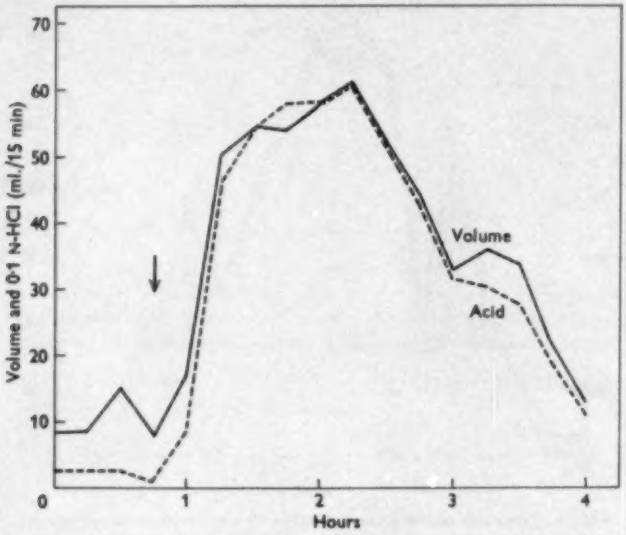

Fig. 7. Response of the human subject to an intramuscular injection (at arrow) of gastrin equivalent to 200 g mucosa (same preparation as Fig. 6).

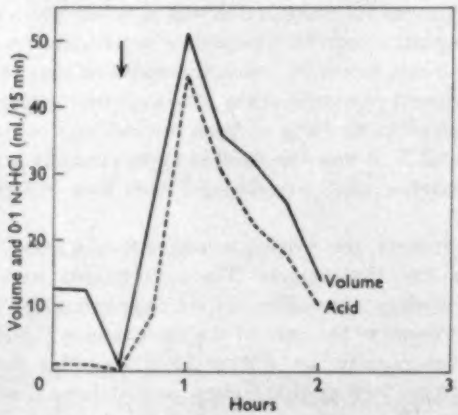

Fig. 8. Response of the human subject to a rapid intravenous injection (30 sec, at arrow) of gastrin equivalent to 200 g mucosa (same preparation as Figs. 6 and 7).

pain than a similar volume of saline solution, and there was no flushing at the site of subcutaneous injection.

In all experiments a very considerable secretory response ensued. After subcutaneous and intramuscular injection secretion began within 15 min and reached a maximum in about 45–60 min (Figs. 6 and 7). After intravenous injection (Figs. 8 and 10) the response began more promptly, the maximum was reached sooner (about 30 min), and the response came to an end more rapidly, i.e. after 1½–2 hr. After subcutaneous or intramuscular injection the maximal secretory rate attained was approximately 7·5 m-equiv HCl (total) in 15 min; after intravenous injection it was

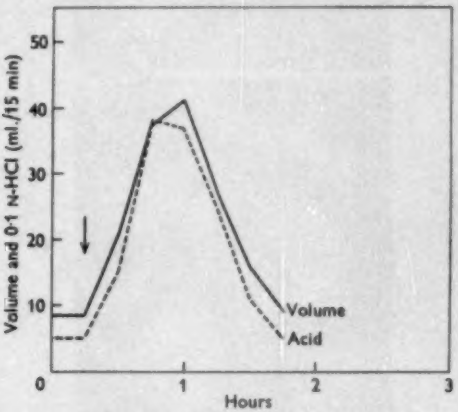

Fig. 9. Response of the human subject to a subcutaneous injection (at arrow) of 1 mg histamine acid phosphate.

rather lower (4·5 and 6·7 m-equiv total HCl in 15 min). These responses may be compared with that given by the same subject to the subcutaneous injection of 1 mg histamine acid phosphate (Fig. 9); the maximal rate of HCl (total) output was 3·8 m-equiv in 15 min.

In a number of samples which were titrated electrometrically to pH 3·5 for determination of free acidity this was found to approximate closely at the peak of the response to the value obtained for total acidity. In several of the experiments the pepsin concentration in the samples of gastric juice was determined, and it was apparent that there was no significant stimulation of pepsin output; the concentration of pepsin in the samples fell to a low level as soon as secretion became established (Fig. 6).

The effect of atropine. In one experiment an intramuscular injection of gastrin was preceded by an intramuscular injection of 1·2 mg atropine sulphate given 30 min earlier. The response to gastrin was almost

suppressed, compared with that given on another occasion to an intramuscular injection of the same amount of material from the same batch of gastrin (Fig. 10). In this experiment the degree of atropinization achieved was apparently only mild, since the subject experienced no acceleration of heart rate and no change in vision although salivation was largely, though not completely, suppressed. It seems likely that had the atropine been given intravenously, an even greater degree of inhibition of the subsequent gastrin response might have been observed.

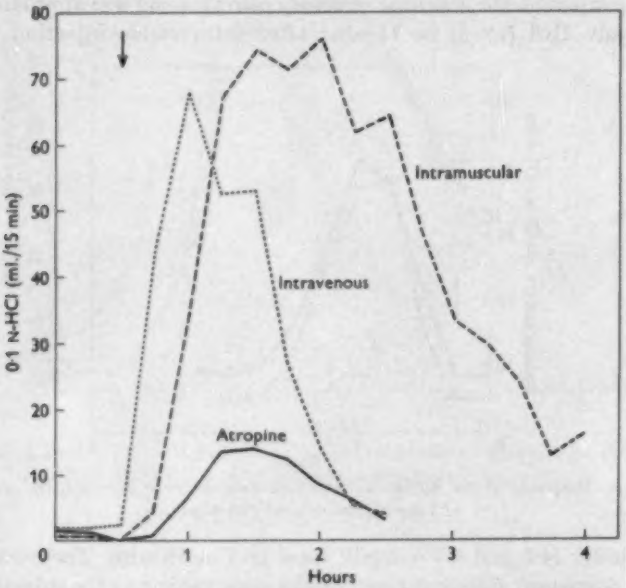

Fig. 10. Responses of the human subject to injections of gastrin (equivalent to 200 g mucosa, at arrow) from the same preparation: (*a*) intramuscularly, (*b*) intravenously (during 5 min), and (*c*) intramuscularly, 30 min after an intramuscular injection of 1·2 mg of atropine.

Histamine content of the preparation

The histamine-like activity present in the final product, as shown by tests on the guinea-pig ileum, was negligible; it amounted to about 0·01 μg/ml. or less. This is attributable to

(1) the precipitation by salt saturation and washing in Stage I, which has been found to remove the bulk of the histamine present in the original crude aqueous extract;

(2) the two precipitations with trichloroacetic acid in Stage I; and

(3) the fact that the calcium phosphate column did not retain histamine;

when a solution containing 100 μg/ml. was passed through it the effluent and washings were found to contain all the histamine originally added.

Nevertheless, in one preparation, 40 mg of histamine acid phosphate was added to the crude aqueous extract; the final preparation, in an amount equivalent to 40 g mucosa, had no action on the blood-pressure of the anaesthetized cat (Fig. 11) but was fully active in stimulating secretion in a conscious dog. This observation also indicates that the action of the active principle on secretion was not due to the liberation of histamine after entry into the circulation.

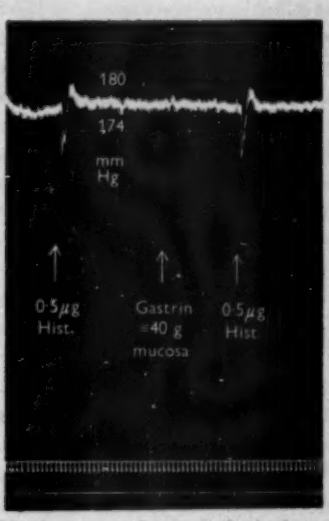

Fig. 11. Effect on the blood-pressure of an anaesthetized cat of (*a*) 0·5 μg histamine base, and (*b*) gastrin equivalent to 40 g mucosa. Time-marker, 10 sec.

Distribution of activity

Preparations made from the corpus region of the hog stomach were found to be inactive; but a small amount of gastrin was present in extracts of the cardiac region. Preparations made from hog duodenum were also inactive. No studies have been made on mucosa obtained from species other than the hog.

General properties

There is ample evidence both from the present work and from previous studies that gastrin is stable in acid solutions but little previous study has been made of its sensitivity to alkali. During the development of this method it was established that gastrin was unaffected by heating to 75° C for 30 min at pH 8·5, or by exposure to pH 10 at room temperature for

15 min. Uvnäs (1943*b*) has reported that gastrin is destroyed by boiling in N/10-NaOH for a short time.

Gastrin activity was found to be dialysable through cellophane, although a considerable loss occurred, probably by adsorption on the walls of the sac. A similar observation was reported by Uvnäs (1943*a*). The failure of Harper (1946) and Jorpes *et al.* (1952) to confirm it is perhaps due to adsorption of gastrin on denatured protein present in their preparations. The finding that gastrin is dialysable is in conformity with its ready absorption on subcutaneous injection.

Some preliminary studies have been made of the behaviour of the final product of the method when subjected to paper electrophoresis, using an apparatus of the type described by Durrum (1951) and applying a solution equivalent to 1 kg mucosa as a streak on a wide paper (Whatman No. 31). Electrophoresis was carried out at room temperature during the night in phosphate and acetic acid buffers. At the end of a run, longitudinal strips were cut from the moist paper, and dried for staining with ninhydrin or by the Cl-imino method (Rydon & Smith, 1952). The regions which stained by either method were then cut from the rest of the paper and eluted with saline for testing. It was found that the activity behaved as though it had an iso-electric point between pH 5 and 6; three or four other inactive substances were also present.

DISCUSSION

The method described in this paper provides a potent and highly purified preparation of a substance which is probably identifiable with the antral hormone; like the latter, it stimulates the secretion of gastric acid but not of pepsin. In the hog stomach it is largely confined to the pyloric region, although a small amount has been found in extracts of the cardiac region. This is the first confirmation of the observation originally made by Edkins (1905). No gastrin activity whatever could be detected in extracts made from hog duodenum by the method described. Komarov (1938), Uvnäs (1945*b*) and Harper (1946) reported that their methods would extract a small amount of gastrin-like activity from hog duodenal mucosa, but they did not study it further.

The preparation described is highly effective in stimulating secretion when injected subcutaneously, as well as intramuscularly or slowly intravenously. It is much less effective when given as a single intravenous dose, particularly in dogs provided with denervated fundic pouches. In this respect gastrin resembles histamine; it is well known that the latter is almost without action on gastric secretion when given as a single intravenous dose, although the same amount injected subcutaneously, intramuscularly or slowly intravenously is highly effective.

No previous studies have been made of the action of gastrin in the human subject. Those reported here show that it stimulates the secretion of acid, but not of pepsin, on subcutaneous, intramuscular or intravenous injection. As in the dog, a single intravenous dose is much less effective than subcutaneous or intramuscular administration, and the response is strongly inhibited by atropine.

The inhibitory effect of atropine on gastrin responses, both in conscious dogs and the human subject, may be considered to provide support for the view that concurrent cholinergic excitation is an important factor determining the response of the oxyntic cell to gastrin. The observation of Komarov (1942*b*) that the action of his gastrin preparation in anaesthetized cats was unaffected by large doses of atropine may perhaps be attributed to a suppression by the anaesthetic of tonic vagal activity present in the conscious animal; but it is perhaps noteworthy that no confirmation of Komarov's observation has been reported.

It has seemed premature to undertake any biochemical studies on the composition of the Stage III product, since more than one substance is present; but the available information suggests that gastrin is a peptide having an iso-electric point in the region of pH 5·5. The preparation contains traces of pigment and three or four unidentified substances detected on paper electrophoresis. The Stage I material has slight secretin activity, but this is absent from the Stage III material. Some preparations have caused slight increase in tone of the denervated fundic pouch when injected as single large intravenous doses in the conscious dog and this is perhaps the basis of the transient 'sinking' feeling experienced by the human subject during rapid intravenous injection of the preparation.

The method described here is not difficult to operate, once some experience of the various procedures has been acquired; it has been used routinely for several months for the processing of several kilograms of antral mucosa weekly and has proved quite reliable. It is, however, unsuitable for use on a larger scale, such as would be required for intensive study of the chemical nature of gastrin, or extensive clinical use. Its merit lies in the fact that it makes available a potent and purified preparation of gastrin which is suitable not only for physiological studies in conscious animals and humans, but also for further investigation of the biochemical properties of the substance, and it is to be hoped that the latter will before long enable a superior method of preparation to be devised.

SUMMARY

1. A new method is described for the preparation from hog antral mucosa of the hormone gastrin in a potent and highly purified state.

2. No gastrin could be extracted from hog duodenal mucosa by this method but extracts of hog cardiac mucosa contained a small amount.

3. Gastrin was found to be dialysable through cellophane, and on paper electrophoresis behaved as though it had an iso-electric point at pH 5–6.

4. The extract strongly stimulated the secretion of gastric acid, but not of pepsin, when injected subcutaneously, intramuscularly or intravenously into conscious dogs and into one human subject.

5. Atropine strongly inhibited the response to gastrin, both in conscious dogs and the human subject.

6. The extract contained negligible amounts of histamine, was free from secretin and was without significant side effects when injected intravenously into the human subject in a dose equivalent to 200 g of mucosa.

We wish to correct a misprint which occurred in the preliminary publication by Gregory & Tracy (1959*a*). In lines 24–25, '3·03 m-equiv/l. HCl' should read '6·03 m-equiv HCl'.

REFERENCES

Barger, G. & Dale, H. H. (1910–11). β-iminazolylethylamine, a depressor constituent of intestinal mucosa. *J. Physiol.* **41**, 499–503.

Dale, H. H. & Laidlaw, P. P. (1910–11). The physiological action of β-iminazolylethylamine. *J. Physiol.* **41**, 318–344.

Durrum, E. L. (1951). Paper ionophoresis. *Science*, **113**, 66–68.

Edkins, J. S. (1905). On the chemical mechanism of gastric secretion. *Proc. Roy. Soc.* B, **76**, 376.

Edkins, J. S. (1906). The chemical mechanism of gastric secretion. *J. Physiol.* **34**, 133–144.

Gavin, G., McHenry, E. W. & Wilson, M. J. (1933). Histamine in canine gastric tissues. *J. Physiol.* **79**, 234–238.

Gregory, R. A. (1955). A new method for the preparation of urogastrone. *J. Physiol.* **129**, 528–546.

Gregory, R. A. (1958). Gastric secretory responses after portal venous ligation. *J. Physiol.* **144**, 123–137.

Gregory, R. A. & Tracy, H. J. (1959*a*). The action of enterogastrone on gastric secretion. *J. Physiol.* **149**, 58–59*P*.

Gregory, R. A. & Tracy, H. J. (1959*b*). The preparation and properties of gastrin. *J. Physiol.* **149**, 70–71*P*.

Gregory, R. A. & Tracy, H. J. (1960). The further purification of gastrin: secretory responses in a human subject. *J. Physiol.* **154**, 52–53*P*.

Gregory, R. A., Tracy, H. J., French, J. M. & Sircus, W. (1960). Extraction of a gastrin-like substance from a pancreatic tumour in a case of Zollinger–Ellison Syndrome. *Lancet*, **278**, 1045–1048.

Grossman, M. I. (1950). Gastrointestinal hormones. *Physiol. Rev.* **30**, 33–90.

Harper, A. A. (1946). The effect of gastric and intestinal extracts of mucosa on the secretion of HCl by the cat's stomach. *J. Physiol.* **105**, 31*P*.

Hunt, J. N. (1948). A method for estimating peptic activity in gastric contents. *Biochem. J.* **42**, 104–109.

Ivy, A. C. (1941). The mechanisms of gastric secretion. *Surgery*, **10**, 861–878.

Ivy, A. C. & Greengard, H. (1938). The isolation of secretin. *Amer. J. Physiol.* **124**, 427–434.

Jorpes, J. E., Jalling, O. & Mutt, V. (1952). A method for the preparation of gastrin. *Biochem. J.* **52**, 327–328.

Komarov, S. A. (1938). Gastrin. *Proc. Soc. exp. Biol., N.Y.*, **38**, 514–516.

KOMAROV, S. A. (1942*a*). Studies on gastrin. I. Methods of isolation of a specific gastric secretagogue from the pyloric mucous membrane and its chemical properties. *Rev. canad. Biol.* **1**, 191–205.

KOMAROV, S. A. (1942*b*). Studies on gastrin. II. Physiological properties of the specific gastric secretagogue of the pyloric mucous membrane. *Rev. canad. Biol.* **2**, 377–401.

LIM, R. K. S. (1922). The question of a gastric hormone. *Quart. J. exp. Physiol.* **13**, 79–103.

LIM, R. K. S., LING, S. M. & LIU, A. C. (1934). Depressor substances in extracts of the intestinal mucosa. Purification of enterogastrone. *Chin. J. Physiol.* **8**, 219–236.

LINDE, S. (1950). Studies on the stimulation mechanism of gastric secretion. *Acta physiol. scand.* **21**, Suppl. 74.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MELLANBY, J. (1932). Secretin. *Proc. Roy. Soc.* B, **111**, 429–436.

MUNCH-PETERSEN, J., RONNOW, G. & UVNÄS, B. (1944). Further studies on the gastric secretory excitant from the pyloric mucosa. *Acta physiol. scand.* **7**, 289–302.

POPIELSKI, L. (1919). β-imidazolyläthylamin und die Organextrakte. I. β-imidazolyläthylamin als mächtiger erreger der Magendrüsen. *Pflüg. Arch. ges. Physiol.* **178**, 214–259.

RYDON, H. N. & SMITH, P. W. G. (1952). A new method for the detection of peptides and similar compounds on paper chromatograms. *Nature, Lond.*, **169**, 922–923.

SACKS, J., IVY, A. C., BURGESS, J. P. & VANDOLAH, J. E. (1932). Histamine as the hormone for gastric secretion. *Amer. J. Physiol.* **101**, 331–338.

UVNÄS, B. (1943*a*). The gastric secretory excitant from the pyloric mucosa. *Acta physiol. scand.* **6**, 97–107.

UVNÄS, B. (1943*b*). Some chemical properties of the gastric secretory excitant from the pyloric mucosa. *Acta physiol. scand.* **6**, 117–122.

UVNÄS, B. (1945*a*). Further attempts to isolate a gastric secretory excitant from the pyloric mucosa of pigs. *Acta physiol. scand.* **9**, 296–305.

UVNÄS, B. (1945*b*). The presence of a gastric secretory excitant in the human gastric and duodenal mucosa. *Acta physiol. scand.* **10**, 97–101.

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TUBULAR REABSORPTION OF PROTEIN IN RATS WITH EXPERIMENTAL PROTEINURIA

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There is evidence that in the rat the glomerular filtrate contains a small amount of plasma protein (Walker, Bott, Oliver & MacDowell, 1941), and that some of this protein is reabsorbed by the cells of the proximal convoluted tubules (see review by Rather, 1952). The observation by Dock (1942) and by Gilson (1949) that, when plasma proteins in the rat were labelled with the dye T-1824 (Evans Blue), a selective accumulation of dye occurred in the cells of the proximal convoluted tubules has led to a method which allows quantitative information about the rate of tubular reabsorption of protein to be obtained. Sellers, Griggs, Marmorston & Goodman (1954) have given a value for the total rate of reabsorption of protein; they suggest that some 90% of filtered protein is reabsorbed by the tubules, the rest appearing as a small but constant proteinuria. These results were confirmed by Mendel (1959) who also showed that, in the rat, both albumin and globulin are reabsorbed by the tubules.

There is, however, a lack of quantitative data relating filtered protein to reabsorbed protein in the presence of increased protein loads. This is a matter of interest not merely in the normally functioning kidney but more particularly in nephrotic states. In the present work a nephrotic state was induced in rats, by injection of nephrotoxic serum (Heymann & Lund, 1948), the effect of which was to produce glomerular lesions without any demonstrable tubular defect. The animals thus treated manifested gross proteinuria and there was, as will be shown, an increased glomerular filtration of protein. The object was to investigate the quantitative relationship between increased protein loads and the tubular reabsorption of protein.

METHODS

The method for determining the rates of albumin and globulin reabsorption is based on the observation of Rawson (1943) that T-1824 in low concentration attaches itself to plasma albumin, and then, as the concentration of the dye is increased, to plasma globulin. It has been shown (Mendel, 1959) that it is possible, by the use of three increasing doses of T-1824,

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to obtain labelling of albumin alone, albumin plus α and β globulin, and finally γ globulin in addition. The rate of reabsorption of the three fractions can be calculated from the concentration of T-1824 attached to them and the T-1824 taken up by the kidneys.

Nephrotoxic serum. An anti-rat kidney serum was produced after the method of Heymann & Lund (1948). Saline-perfused, whole-rat-kidney homogenate was given in 0·5 g doses intra-peritoneally to 2 kg rabbits twice weekly for 3 weeks. The course of injections was repeated three times with weekly intervals between them. The rabbits were exsanguinated 6 days after the last injection and the serum was stored by deep freezing.

Experimental proteinuria. Wistar strain male rats of average weight 200 g (range 195–205 g) and fed on a diet containing 14 % protein (diet 41) were used. Experimental proteinuria was induced by the intravenous injection of 0·3 ml. of nephrotoxic serum through an indwelling polyethylene cannula in an external jugular vein. A marked increase in proteinuria usually occurred within 24 hr. In some animals a second intravenous injection was required on the next day. Histological examination of the kidneys on the third day after the onset of proteinuria showed either normal histology or, at the most, some thickening of the basement membranes in the glomeruli. The cells of the proximal convoluted tubules appeared to be normal.

Operative procedure. The methods used in the present work have been described previously in detail (Mendel, 1959). All experiments were carried out on the third day after the onset of proteinuria. An injection of T-1824 was given intravenously, and after a 3 hr period, during which the animal was deprived of food and water, the carotid artery was cannulated under ether anaesthesia and blood collected into a dry centrifuge tube. The kidneys were washed free of blood by perfusion with 0·9 % sodium chloride solution and removed for analysis.

Estimation of renal T-1824. The perfused kidneys were decapsulated and the renal pelves cut away; they were then homogenized in 6 ml. distilled water in a standard 'universal container' which fitted a tissue homogenizer (M.S.E.). In most experiments (thirty) the supernatant above the settled homogenate was colourless, indicating adequate perfusion. In eight experiments there was T-1824 colouring of the supernatant because of ineffective perfusion; this was usually associated with heavy proteinuria and may have been due to difficulty in washing out dye-stained protein in the capsular space and tubules. These experiments were rejected. T-1824 was estimated in the way previously described (Mendel, 1959).

Estimation of serum T-1824. The method used to determine the concentration of T-1824 attached to individual serum proteins was also described in detail in the previous paper (Mendel, 1959). A concentration of anhydrous Na_2SO_4 of 23·5 g/100 ml. was used to precipitate total globulin, and 17·75 g/100 ml. to precipitate γ globulin. 0·2 ml. of serum was added to 3 ml. volumes of 0·9 % NaCl and of 23·5 and 17·75 % solutions of anhydrous Na_2SO_4 in centrifuge tubes. After mixing, 1·5 ml. of ether was added to the tubes containing Na_2SO_4; these were stoppered, mixed and centrifuged. The T-1824 content of the supernatants was determined after suitable dilution with distilled water.

Serum proteins. Samples of sera from individual rats were fractionated with anhydrous Na_2SO_4 as described above, and the protein content of the supernatant determined by the micro-Kjeldahl method.

Urinary proteins. 24 hr volumes of urine, measured to within 0·1 ml., were obtained from rats in metabolism cages with free food and water. Total protein was determined by the biuret method of Kingsley (1939) on the protein precipitated with 10 % trichloroacetic acid. The qualitative determination of urinary protein was made by paper electrophoresis after the method of Flynn & de Mayo (1951). Whatman No. 1 paper was used in 5 × 36 cm strips. A barbitone buffer of pH 8·6 and ionic strength 0·075 M with a constant current of 1·0 mA per strip for 18 hr gave good separation. The strips were stained with Azocarmine B and evaluated by scanning and planimetry.

Inulin clearance. The method of Dicker (1949) was used to determine the inulin clearance. Inulin in blood and urine was measured by the method of Roe, Epstein & Goldstein (1949).

Throughout this paper, mean values are given together with the standard error of the mean.

RESULTS

Serum albumin and proteinuria. The effect of proteinuria on the serum albumin was determined in ten rats with a wide range of experimental proteinuria. Table 1 shows the proteinuria before treatment with nephrotoxic serum, and the proteinuria and serum albumin on the third day after the onset of proteinuria following nephrotoxic serum. It can be seen that rats with proteinuria greater than 8 mg/hr had serum albumins below 2 g/100 ml.; in ten normal rats the mean serum albumin was $3{\cdot}1 \pm 0{\cdot}06$ g/100 ml.

TABLE 1. The relationship of proteinuria to serum albumin in ten rats. The values after treatment were obtained on the third day after the onset of increased protein excretion

Before treatment. Proteinuria (mg/hr)	After treatment	
	Proteinuria (mg/hr)	Serum albumin (g/100 ml.)
0·8	2·7	2·8
0·4	3·0	2·5
0·4	4·2	2·4
0·2	5·7	2·7
0·6	6·3	2·0
0·3	6·6	2·6
0·6	8·0	2·0
0·5	11·0	1·5
0·2	13·2	1·6
0·7	17·0	1·0

Dosage of T-1824 and its attachment to serum proteins. In experiments with normal rats doses of T-1824 which proved suitable for the progressive labelling of serum proteins were 2 mg/100 g rat, to label albumin; 5 mg/100 g rat, to label albumin plus α and β globulin, and 10 mg/100 g rat, to label all the serum proteins (Mendel, 1959). Albumin binds the greatest proportion of the dye. In rats with experimental proteinuria it seemed likely that the lowering of serum albumin might cause such a reduction of T-1824 binding power that the dosage of T-1824 previously used might not be suitable; doses of 2 mg/100 g rat might cause a significant labelling of α and β globulin and 5 mg/100 g rat might similarly cause labelling of γ globulin. Preliminary experiments were designed to investigate this possibility.

Rats with proteinuria greater than 8 mg/hr usually had oedema and ascites, which made the measurement of T-1824 disappearance curves extremely difficult. Consequently, experiments to determine the most suitable doses of T-1824 were carried out on eight rats with a mean

proteinuria of 7·0 ± 0·17 mg/hr. Doses of T-1824 suitable under these conditions were regarded as suitable in the presence of less-marked proteinuria.

T-1824 in the dosage of 1 mg/100 g rat, as compared with 2 mg/100 g in normal rats, was the smallest dose of dye which allowed measurable amounts of T-1824 to be recovered from the kidneys. With this dose of dye over 90% of the total dye was attached to albumin in blood samples taken 5 min after injection. Doses of T-1824 of 5 mg/100 g rat were found to label γ globulin, the total content of dye in the serum being greater than that in the supernatant after fractionation with 17·75% Na_2SO_4. Doses of T-1824 of 4 mg/100 g rat, however, did not cause significant labelling of γ globulin in samples of blood taken 5 min after injection, and were therefore used to label albumin plus α and β globulin. The dose of dye used to label all the serum proteins was kept at 10 mg/100 g rat.

Time concentration curves of T-1824 in serum. The rate of disappearance from the serum of the T-1824 attached to individual protein fractions was estimated in twenty-five rats with each dose of T-1824 (Figs. 1 and 2). The values at each interval of time are the means of five individual results, obtained with rats chosen so that the average proteinuria at each time interval was between 3 and 5 mg/hr. There was a similar proteinuria in the group of rats in which the renal uptake of T-1824 was determined.

Figure 1*A* shows the values obtained with doses of T-1824 of 1 mg/100 g rat, and Fig. 1*B* the values with 4 mg/100 g rat. The concentration of T-1824 attached to α and β globulin with the dose of T-1824 of 1 mg/100 g rat was less than 1 mg/100 ml. at any time interval and is not represented in the figure. Figure 2*A* shows the fall in concentration of T-1824 attached to albumin and α and β globulin with the dose of T-1824 of 10 mg/100 g rat, and Fig. 2*B* the fall of concentration of T-1824 attached to γ globulin.

Renal uptake of T-1824

The renal content of T-1824 was estimated in thirty rats, on the third day after the onset of increased proteinuria, and 3 hr after the intravenous injection of T-1824. Groups of ten rats were used with each dose of T-1824 and were chosen so that the mean proteinuria for each group was about 4 mg/hr. Table 2 shows the individual results for renal T-1824, the mean proteinuria and the mean serum albumin level in each group.

The mean values of renal T-1824 shown in Table 2 were greater than those found in normal rats used as controls. Thus the mean value of renal T-1824 with doses of T-1824 of 1 mg/100 g rat was of the same order as the mean value of 30 ± 2·4 μg T-1824 obtained in ten normal rats with doses of T-1824 of 2 mg/100 g rat. The mean value with doses of T-1824

of 4 mg/100 g rat was significantly greater ($P < 0.01$) than the 88 ± 4.2 μg T-1824 obtained in ten normal rats with doses of T-1824 of 5 mg/100 g rat, and the mean value with doses of T-1824 of 10 mg/100 g

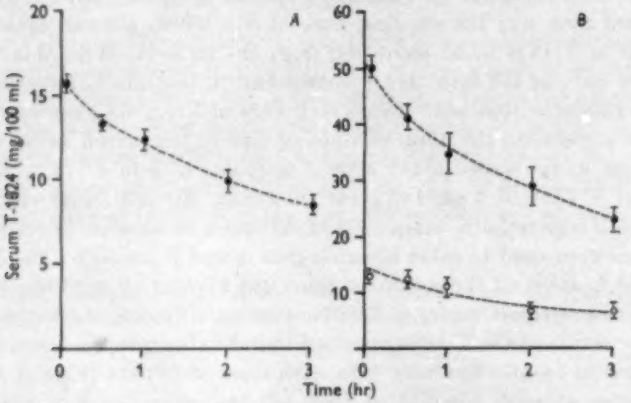

Fig. 1. The rate of fall of concentration of serum T-1824 following intravenous injection of T-1824 in rats with experimental proteinuria. Dose of T-1824 in *A*, 1 mg/100 g rat; in *B*, 4 mg/100 g. The values at each interval of time are the means of experiments in five rats. Vertical lines show S.E. of means. ● T-1824 attached to albumin; ○ T-1824 attached to α and β globulin.

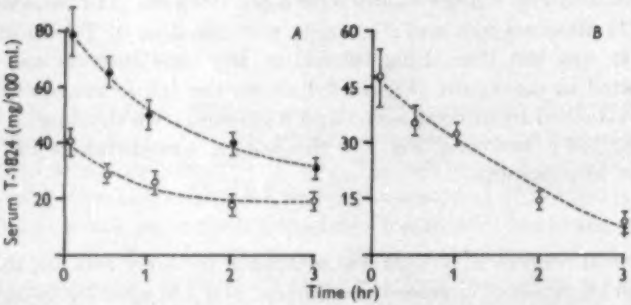

Fig. 2. The rate of fall of concentration of serum T-1824 in twenty-five rats following an intravenous injection of 10 mg T-1824/100 g rat. The values at each interval of time are the means of experiments in five rats: *A*, amount of T-1824 attached to albumin and α and β globulin; convention as in Fig. 1. *B*, T-1824 attached to γ globulin.

rat was significantly greater ($P < 0.01$) than the 333 ± 20 μg T-1824 obtained in ten normal rats with similar doses of T-1824.

It has been shown previously (Mendel, 1959) that, when doses of T-1824 of 10 mg/100 g rat were given to normal rats, an amount of renal T-1824

could be recovered 2 min after injection which could not be explained on the basis of tubular reabsorption of protein, but appeared to be due to free T-1824 entering the cells of the proximal tubules as such. In six experiments on rats with experimental proteinuria, the kidneys were perfused within 2 min of giving an intravenous injection of T-1824 of 10 mg/100 g rat. The renal T-1824 recovered was 116 ± 6 μg, a value significantly greater ($P < 0{\cdot}01$) than the 33 ± 2 μg T-1824 recovered from six normal control rats. Similar experiments with doses of T-1824 of 1 and 4 mg/100 g rat did not show any initial rapid uptake of dye by the kidneys.

TABLE 2. Renal content of T-1824 (μg) in individual rats 3 hr after the intravenous injection of the doses of T-1824 shown, and the mean proteinuria and the mean serum albumin levels for each group

Dose of T-1824	1 mg/100 g rat	4 mg/100 g rat	10 mg/100 g rat
	23	211	730
	33	200	730
	28	182	475
	57	168	645
	19	160	484
	41	121	925
	33	150	606
	26	230	378
	46	149	960
	28	190	500
Means ± S.E.	$33 \pm 3{\cdot}6$	176 ± 10	643 ± 61
Mean proteinuria (mg/hr)	$4{\cdot}6 \pm 0{\cdot}8$	$3{\cdot}9 \pm 0{\cdot}6$	$3{\cdot}8 \pm 0{\cdot}6$
Mean serum albumin (g/100 ml.)	$2{\cdot}5 \pm 0{\cdot}1$	$2{\cdot}5 \pm 0{\cdot}1$	$2{\cdot}4 \pm 0{\cdot}1$

Calculation of tubular reabsorption of protein

The rate of tubular reabsorption of protein has been calculated from the serum proteins, the renal T-1824 and the mean value for T-1824 attached to individual serum proteins during the 3 hr period, which has been calculated from the area under the curves in Figs. 1 and 2. The mean serum protein values in the thirty rats in which the renal uptake of T-1824 was determined were: albumin $2{\cdot}5 \pm 0{\cdot}08$, α and β globulin $1{\cdot}7 \pm 0{\cdot}07$ and γ globulin $1{\cdot}4 \pm 0{\cdot}09$ g/100 ml. The other data are shown in Table 3.

Albumin. The mean value for renal T-1824 of $33 \pm 3{\cdot}6$ μg, obtained with the dose of T-1824 of 1 mg/100 g rat, is considered for the purpose of calculation to represent only albumin reabsorption, the attachment of T-1824 to α and β globulin being very small. From Table 3 it can be shown that there was $11 \pm 0{\cdot}5$ mg of T-1824 attached to $2{\cdot}5 \pm 0{\cdot}08$ g of serum albumin. The amount of albumin attached to the $33 \pm 3{\cdot}6$ μg of T-1824 recovered from the kidneys was therefore $7{\cdot}5 \pm 0{\cdot}8$ mg; this is albumin reabsorbed in 3 hr, representing a rate of albumin reabsorption of $2{\cdot}5 \pm 0{\cdot}3$ mg/hr.

α and β globulin. The value for renal T-1824 of 176 ± 10 μg represents albumin plus α and β globulin reabsorption (Table 3). Before the rate of α and β globulin reabsorption can be calculated it is necessary to calculate the renal T-1824 accounted for by albumin. The mean value for T-1824 attached to 2·5 ± 0·08 g of albumin under these conditions was 34 ± 3·3 mg, so that 1 mg of albumin was attached to 13·6 ± 1·3 μg of dye. Since the amount of albumin reabsorbed during the 3 hr period was 7·5 ± 0·8 mg, the amount of T-1824 accounted for by albumin was 102 ± 14 μg. The renal T-1824 accounted for by α and β globulin reabsorption was the remaining dye, i.e. 74 ± 17 μg, and as 9 ± 0·9 mg of T-1824 was attached to 1·7 ± 0·07 g of α and β globulin, the protein represented by 74 ± 17 μg of dye was 13·9 ± 3·2 mg, giving a rate of α and β globulin reabsorption of 4·6 ± 1·1 mg/hr.

TABLE 3. Renal T-1824 3 hr after intravenous injection of T-1824, and the mean concentration of T-1824 attached to serum protein fractions during the 3 hr after intravenous injection of T-1824

Dose of T-1824 (mg/100 g rat)	Renal T-1824 (μg)	Mean T-1824 attached to serum proteins (mg/100 ml.)		
		Albumin	α + β globulin	γ globulin
1	33 ± 3·6	11 ± 0·5	—	—
4	176 ± 10	34 ± 3·3	9 ± 0·9	—
10	643 ± 61	50 ± 5·5	23 ± 3·2	34 ± 2·1

γ globulin. The value of 643 ± 61 μg of renal T-1824 (Table 3) represents reabsorption of all the serum protein fractions. The amount of T-1824 accounted for by albumin and α and β globulin reabsorption has been calculated as explained above, from the serum protein values, the mean concentration of T-1824 attached to them, and the rate of reabsorption. Thus albumin accounted for 150 ± 23 μg T-1824, and α and β globulin 188 ± 49 μg T-1824. In addition 116 ± 6 μg of renal T-1824 has been accounted for by free dye. The amount of T-1824 accounted for by γ globulin was thus 189 ± 82 μg, and as 34 ± 2·1 mg of T-1824 was attached to 1·4 ± 0·09 g of γ globulin, 189 ± 82 μg of T-1824 was attached to 7·7 ± 3·4 mg of γ globulin, representing a rate of γ globulin reabsorption of 2·5 ± 1·1 mg/hr.

Proteinuria and tubular reabsorption of protein

Proteinuria. Electrophoresis of urinary protein was carried out on twenty individual urines obtained from rats in which renal T-1824 was subsequently measured. Urines were collected for a period of 24 hr immediately before giving the dye. Figure 3 shows the results of electrophoresis of serum and urine obtained from a rat 3 days after the onset of increased proteinuria. It can be seen that albumin, and α_1, β and γ

globulin appeared in the urine. The mean rate of proteinuria in the twenty rats was $4{\cdot}2 \pm 0{\cdot}5$ mg/hr. The mean rates of excretion of individual serum proteins were: albumin $1{\cdot}8 \pm 0{\cdot}2$ mg/hr, α globulin $1{\cdot}0 \pm 0{\cdot}22$ mg/hr, β globulin $0{\cdot}9 \pm 0{\cdot}12$ mg/hr and γ globulin $0{\cdot}5 \pm 0{\cdot}07$ mg/hr.

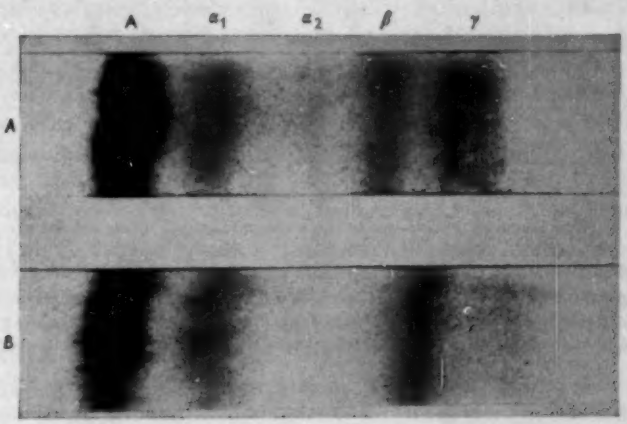

Fig. 3. Electrophoresis of serum and urine proteins of a rat with experimental proteinuria. *A*, serum; *B*, urine. Rate of proteinuria 4·2 mg/hr.

Proteinuria and renal T-1824. The relationship of the renal uptake of T-1824 to the rate of proteinuria in thirty rats is shown in Fig. 4. Figure 4*A* shows the renal uptake of T-1824-labelled albumin and the rate of urinary albumin excretion, Fig. 4*B* the renal uptake of T-1824-labelled albumin and α and β globulin and the rate of excretion of these fractions in the urine, and Fig. 4*C* the renal uptake of T-1824 with all the serum proteins labelled and the total rate of protein excretion. It can be seen that there was no clear relationship between the reabsorption of labelled protein and its rate of excretion. The correlation coefficient for the data shown in Fig. 4*A* was 0·6, which is not significant at 5% level for a sample of this size.

Proteinuria and G.F.R. The relationship of proteinuria and glomerular filtration rate, determined by inulin clearance, was determined in ten rats 3 days after the onset of increased proteinuria. The mean rate of proteinuria in the group was $4{\cdot}7 \pm 0{\cdot}7$ mg/hr and the mean inulin clearance $0{\cdot}49 \pm 0{\cdot}02$ ml./100 g rat/min, a value which did not differ significantly from the $0{\cdot}47 \pm 0{\cdot}02$ ml./100 g rat found in ten normal rats. Figure 5 shows proteinuria plotted against inulin clearance in the individual rats. It can be seen that these were not related.

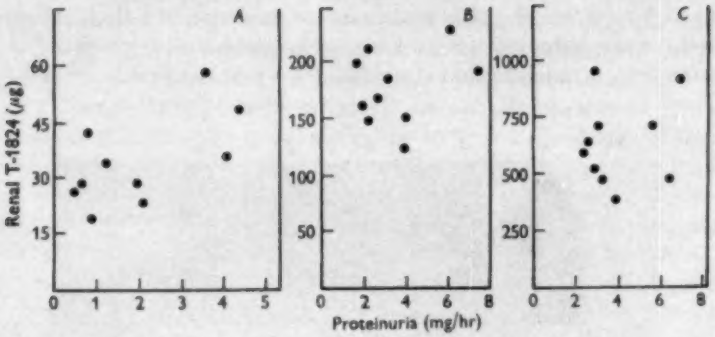

Fig. 4. The relation of proteinuria to the renal uptake of T-1824-labelled proteins in thirty rats with experimental proteinuria. *A*, dose of T-1824, 1 mg/100 g rat; renal uptake of labelled albumin and urine albumin excretion. *B*, dose of T-1824, 4 mg/100 g rat; renal uptake of labelled albumin plus α and β globulin, and urinary albumin plus α and β globulin excretion. *C*, dose of T-1824 10 mg/100 g rat; renal uptake of T-1824 with all serum protein labelled and total proteinuria.

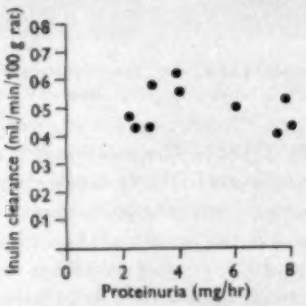

Fig. 5. The relation of proteinuria to inulin clearance in ten rats with experimental proteinuria.

DISCUSSION

In the present work rats were used in which proteinuria had been induced by the use of a nephrotoxic serum. It is known that the primary lesion induced by such means is in the glomerulus (Ortega & Mellors, 1956) and that the kidneys may show minimal histological changes or severe degrees of glomerular and tubular damage. The more severely affected animals have oedema and ascites in varying degrees, and the syndrome has been likened to the equivalent pathological state in man (Heymann & Lund, 1948). It was unfortunately not possible in the present work to use animals with gross oedema and ascites. Nevertheless, it is reasonable

to assume that the animals used were suffering from a pathological process, less advanced, but part of the same syndrome.

The results indicate that there was an increased uptake of T-1824-labelled protein by the kidneys, the values for renal T-1824 being greater than those obtained in normal rats. An increase in the renal uptake of labelled protein has previously been observed by Sellers, Smith, Marmorston & Goodman (1952) using T-1824-labelled protein in rats with renin proteinuria, and by Spector (1954) using ^{131}I-labelled protein in rats with an experimental glomerular lesion of the type produced in the present work. The calculated rate of tubular reabsorption of protein was 9·6 mg/hr, consisting of 2·5 mg/hr albumin, 4·6 mg/hr α and β globulin and 2·5 mg/hr γ globulin. In the normal rat the rate of tubular reabsorption of protein has previously been reported to be of the order of 3·6 mg/hr and the rates of reabsorption of the individual serum proteins as albumin 1·1, α and β globulin 1·4 and γ globulin 1·1 mg/hr (Mendel, 1959). It can be seen that in the present experiments there was a more than twofold increase in tubular reabsorption of protein, and that the increase was shown by each of the labelled serum protein fractions.

The relationship of filtered protein to reabsorbed protein shows that with a G.F.R. of 0·49 ml./min/100 g rat, the total protein filtered for a 200 g rat/hr in 59 ml. filtrate was 13·6 mg (tubular reabsorption 9·6 mg, proteinuria 4 mg) and the concentration of protein in the glomerular filtrate thus about 23 mg/100 ml. Some 70% of the filtered protein was reabsorbed by the tubules. The percentage reabsorption of the individual serum protein fractions, calculated from the rate of reabsorption and the rate of urinary loss, was albumin 58%, α and β globulin 70% and γ globulin 83%.

In the nephrotic animals the uptake of labelled protein by the kidneys did not show any further increase with proteinuria increasing above about 4 mg/hr. It would seem therefore that under the conditions of these experiments tubular reabsorption of protein was maximal. It may well be that some undetected tubular damage was present and it is possible that a completely normal tubule is capable of greater reabsorption. Nevertheless, a rate of reabsorption of 9·6 mg/hr is important in the conservation of plasma protein. If the value of total serum T-1824 taken 5 min after intravenous injection is used for the calculation of plasma volume, values of 11·7, 12·5 and 11·8 ml. are obtained for the three doses of dye. With an average plasma volume of 12 ml. and a total serum protein value of 5·6 g/100 ml., the total circulating protein is of the order of 670 mg. With a rate of tubular reabsorption of protein of 9·6 mg/hr it follows that about one third of the total circulating protein could be reabsorbed by the tubules daily.

SUMMARY

1. Tubular reabsorption of protein has been measured, by use of the dye T-1824, in rats with experimental proteinuria produced by means of a nephrotoxic serum.

2. Renal uptake of T-1824-labelled protein was measured in rats with rates of proteinuria of up to 8 mg/hr and found to be increased.

3. The calculated rate of tubular reabsorption of protein in rats with proteinuria of about 4·0 mg/hr was 9·6 mg/hr. The rates of reabsorption of individual serum protein fractions were: albumin 2·5, α and β globulin 4·6 and γ globulin 2·5 mg/hr.

4. Tubular reabsorption of protein accounted for 70% of the total filtered load. The protein content of the glomerular filtrate was calculated to be about 23 mg/100 ml.

5. Renal uptake of T-1824 was not further increased with increased rates of proteinuria, suggesting that under the conditions of these experiments protein reabsorption was maximal.

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REFERENCES

Dicker, S. E. (1949). Effect of the protein content of the diet on the glomerular filtration rate of young and adult rats. *J. Physiol.* **108**, 197–202.

Dock, W. (1942). Proteinuria and the associated renal changes. *New Engl. J. Med.* **227**, 633–636.

Flynn, F. V. & de Mayo, P. (1951). Micro-electrophoresis of protein on filter paper. *Lancet*, **261**, 235–239.

Gilson, S. B. (1949). Studies on proteinuria in the rat. *Proc. Soc. exp. Biol., N.Y.*, **72**, 608–613.

Heymann, W. & Lund, H. Z. (1948). Lipemic nephrosis in rats. *Science*, **108**, 448–449.

Kingsley, G. R. (1939). The determination of serum total protein, albumin and globulin by the biuret reaction. *J. biol. Chem.* **131**, 197–200.

Mendel, D. (1959). Tubular reabsorption of protein in the rat. *J. Physiol.* **148**, 1–13.

Ortega, L. G. & Mellors, R. C. (1956). The role of localised antibodies in the pathogenesis of nephrotoxic nephritis in the rat. *J. exp. Med.* **104**, 151–169.

Rather, L. J. (1952). Filtration, resorption and excretion of protein by the kidney. *Medicine, Baltimore*, **31**, 357–380.

Rawson, R. A. (1943). The binding of T-1824 and structurally related diazo dyes by plasma proteins. *Amer. J. Physiol.* **138**, 708–717.

Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949). A photometric method for the determination of inulin in plasma and urine. *J. biol. Chem.* **178**, 839–845.

Sellers, A. L., Griggs, N., Marmorston, J. & Goodman, H. C. (1954). Filtration and reabsorption of protein by the kidney. *J. exp. Med.* **100**, 1–9.

Sellers, A. L., Smith, S., Marmorston, J. & Goodman, H. C. (1952). Studies on the mechanism of experimental proteinuria. *J. exp. Med.* **96**, 643–652.

Spector, W. G. (1954). The reabsorption of labelled proteins by the normal and nephrotic rat kidney. *J. Path. Bact.* **68**, 187–196.

Walker, A. M., Bott, P. A., Oliver, J. & MacDowell, M. C. (1941). The collection and analysis of fluid from single nephrons of the mammalian kidney. *Amer. J. Physiol.* **134**, 580–595.

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THE ACCURACY OF DIRECTING THE EYES AND THE HAND IN THE DARK

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Eye movements are probably the most precise muscular movements we make, and no one doubts that the exceptionally large number of sense organs in the eye muscles are somehow involved in making them precise. One simple way in which sense organs might be used would be to supply information about the position of the eyeballs which could be used consciously to correct voluntary efforts to move the eyes, in the same way that we use position sense in the limbs to direct their movements when we are not looking at them. When Sherrington (1897, 1898) discovered sensory receptors in the eye muscles he seems to have taken it for granted that this was how they were used, and he later claimed to have done experiments that showed such a refined appreciation of the posture of the eyeball in the dark as 'must be attributed to muscular sense' (Sherrington, 1918). If the eyes can be positioned very accurately in the dark there would be a strong presumption that accurate positional information was available, for without sense organs to monitor their performance it is not likely that the muscles could be made to give accurately reproducible contractions. It might be as well to mention, however, that if positional information is used it does not follow it is used at a conscious level, as Sherrington apparently thought; Brindley & Merton (1960) have recently given evidence to support Helmholtz's view that the human eyes are without conscious position sense and that, in the absence of visual clues, we only know how far we have moved our eyes by judging the effort of will put into moving them (Helmholtz, 1867, 1925, section 29). On this theory positional information would be used in a lower level automatic nervous mechanism to ensure that the muscles accurately obeyed the effort the subject was making, i.e. in a positional servo. The over-all accuracy attainable with this mechanism ought to be as good as with Sherrington's, for there is no reason why we should not be able to judge the size of motor volleys leaving the brain as accurately as we can judge the size of sensory volleys arriving. Thus measurements of the accuracy of positioning the

eyes in the dark, while they might raise a presumption that positional information is used, could not indicate where.

The present experiments were undertaken from the conviction that the sense organs in muscle are not, in fact, designed to give accurate positional information. Ordinary skeletal muscle contains Golgi tendon organs, signalling muscle tension, and muscle spindles, responding to change of length, but seemingly unsuitable for measuring the absolute length because of their contractile poles. No doubt the absolute length of a muscle could be worked out indirectly, if the rate of discharge of the γ efferents to the muscle spindles were taken into account too, but this is a long way round and not likely to give a very good answer. Eye muscles have a highly specialized structure, but the sense organs they contain appear to fall into the same two categories. Therefore one would not expect that the power of directing the eyes in the dark would be very precise. The experiments of Fick (1905), Sandström (1951) and those cited by Rivers (1900) support this view. Sherrington, however, in the 1918 paper quoted above, claims to have shown the opposite; he gives no reference to the place of publication of his experiments but they are presumably those briefly described in 1898. A full account was then promised but apparently never published. The account of 1898 reads:

'A contribution towards the physiological inquiry into the matter has been made as follows, and has given a clear reply. The conjunctivae, both palpebral and ocular, and the corneae of both eyes have been rendered deeply anaesthetic to cold, warmth, touch, and pain by liberal applications of cocaine. Then in a completely dark room the power to direct the gaze with accuracy in any required direction has been tested. The person under examination is seated, with the head securely fixed, in front of a screen. One of his hands carries a marker. The hand is moved by an assistant, and is made to mark the screen at some one point; it is then passively replaced. The person under observation during this time keeps the eyes open in the primary position or sits with them closed. He is then required to direct his gaze to the spot marked on the screen. The light is then switched on, and the point to which the gaze is turned is noted. The power to direct the gaze under these circumstances has been found to remain good.'

The difficulty in these experiments must have been to note the point to which the gaze was turned when the light came on, and we are not told how this was done. In view of this uncertainty it was decided to re-investigate the question.

In Sherrington's experiment the position of the mark on the screen is known to the subject only through position sense in his arm. What is tested, therefore, involves his knowledge both of his eye position and of his arm position. In the present experiments these two factors have also

been investigated separately, both because it is important to know to what extent the errors in the Sherrington type of experiment are due to the arm, and because it is of general interest to compare the performances of the arm and the eye.

In order to determine the direction of the eyes in darkness recourse was had to after-images. A small circular aperture was illuminated by a brief powerful flash from an electronic flash tube. If the subject was fixating the aperture he obtained an after-image of the aperture at the fixation point; similarly if the fixation point was, say, 2° to the right of the aperture the after-image lay 2° to the left. Since after-images behave as if they are fixed in position on the retina, keeping the same position relative to the fixation point independently of eye movements, the position of the fixation point relative to the aperture at the time of the flash could be determined subsequently by fixating the zero of a scale subtending degrees at the subject's eye and observing the apparent position of the after-image on the scale. If, as in the above example, the after-image were found to lie 2° to the left of the fixation point when looking at the scale, the subject must have been gazing 2° to the right of the aperture when the flash went off.

RESULTS

The ability to fixate points previously located by touch

The first experiment was designed to reproduce the conditions of Sherrington's experiment as closely as could conveniently be managed. The subject bit a dental grip to steady his head. The flash aperture was approximately 57 cm from his eyes (1 cm subtends 1° at this distance) and a little below eye level. The flash gun was mounted on a carriage and could move horizontally along the arc of a circle whose centre lay between the subject's eyes. Nine positions of the carriage were used, numbered 1 to 9. In position 1 the aperture was 20° to the left of the primary (straight-ahead) position of the eyes, in position 2 it was 15° to the left and so on by steps of 5° to position 9 which was 20° to the right. The successive positions of the carriage during the run were taken from a table of random numbers, omitting zeros and selecting runs which had roughly equal numbers of high and low integers. Each observation began with the carriage in the centre (position 5) and the room lights on. The subject shut his eyes and the experimenter then moved the carriage to its next position. The experimenter picked up the subject's right arm from the table, firmly pressed the tip of the index finger into the flash aperture, and replaced the arm. The lights being now put out the subject opened his eyes and attempted to fixate the aperture; when he was ready (5–10 sec

after his finger had left the aperture) he pressed a button to fire the flash. With the lights on he noted the apparent position of the after-image relative to the fixation point and gave both its horizontal (x) and vertical (y) co-ordinates. When, as frequently happened, the after-images from the two eyes were not in the same position, owing to errors of convergence, the mid point between them was taken. In giving results the apparent positions reported by the subject are always converted to true positions of the fixation point relative to the aperture at the time of the flash.

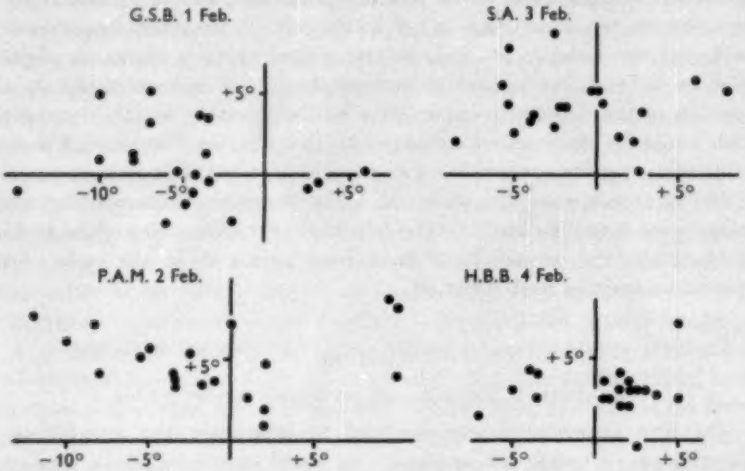

Fig. 1. The scatter of fixation points when attempts were made, in the dark, to fixate a point previously located by touching it with the forefinger. The point touched is the origin.

Horizontal errors to the right are given as + and to the left as −, vertical errors above as + and below as −. Each subject was allowed two or three attempts to get used to the task. After that all results were written down and are reported here. It is a general rule throughout the paper that except for two or three trials all the results obtained with each experimental arrangement are given without selection, unless the contrary is specifically mentioned.

In Fig. 1 are plotted the performances of four subjects, all young male research workers accustomed to psychophysical experiments. The errors made are of the order of 5° with individual attempts up to 15° from the target. Each diagram gives without distinction the individual points from two series of ten observations made by the subject at a single session. The means for the two series separately and the standard deviations of single observations about the means appear in Table 1, in the two columns

TABLE 1. The errors made in attempting to direct the eyes in the dark at a point previously touched (finger-eye columns), or previously seen (eye-eye column) or in attempting to touch a point previously touched (finger-finger column). The horizontal position of the target in all instances randomized as described in the text. In each experiment the number of observations $n = 10$ except that for P. A. M. 16 February $n = 18$. Errors to the left or downwards −, to the right or upwards +. The figures given are the mean horizontal and mean vertical errors ($\bar{x}$ and $\bar{y}$) and the standard deviations of single observations about these means. All values in degrees

Subject and date	Finger-eye (1)		Eye-eye		Finger-finger		Finger-eye (2)	
	$\bar{x}$	$\bar{y}$	$\bar{x}$	$\bar{y}$	$\bar{x}$	$\bar{y}$	$\bar{x}$	$\bar{y}$
P. A. M. 30 Jan.	$-3{\cdot}2 \pm 3{\cdot}9$	$+1{\cdot}8 \pm 1{\cdot}7$	$-2{\cdot}0 \pm 2{\cdot}5$	$-1{\cdot}5 \pm 0{\cdot}7$	—	—	—	—
2 Feb.	$-0{\cdot}8 \pm 5{\cdot}2$	$+3{\cdot}7 \pm 1{\cdot}5$	$-1{\cdot}1 \pm 2{\cdot}1$	$+1{\cdot}2 \pm 0{\cdot}8$	$-0{\cdot}2 \pm 4{\cdot}4$	$+1{\cdot}9 \pm 0{\cdot}9$	$-2{\cdot}0 \pm 7{\cdot}4$	$+5{\cdot}8 \pm 2{\cdot}1$
16 Feb.	$-2{\cdot}5 \pm 5{\cdot}7$	$+2{\cdot}9 \pm 2{\cdot}1$	$-1{\cdot}2 \pm 3{\cdot}1$	$+1{\cdot}3 \pm 1{\cdot}3$	$-3{\cdot}7 \pm 2{\cdot}7$	$+1{\cdot}5 \pm 1{\cdot}3$	—	—
H. B. B. 30 Jan.	$-2{\cdot}7 \pm 4{\cdot}2$	$-0{\cdot}3 \pm 1{\cdot}4$	$-3{\cdot}5 \pm 2{\cdot}2$	$-0{\cdot}4 \pm 2{\cdot}0$	—	—	$-2{\cdot}0 \pm 5{\cdot}1$	$+0{\cdot}7 \pm 4{\cdot}0$
4 Feb.	$+0{\cdot}6 \pm 3{\cdot}5$	$+2{\cdot}5 \pm 1{\cdot}3$	$-1{\cdot}6 \pm 2{\cdot}7$	$-1{\cdot}8 \pm 1{\cdot}1$	$+0{\cdot}7 \pm 1{\cdot}6$	$+0{\cdot}8 \pm 1{\cdot}6$	$+0{\cdot}5 \pm 3{\cdot}3$	$+3{\cdot}4 \pm 1{\cdot}4$
G. S. B. 1 Feb.	$-4{\cdot}6 \pm 4{\cdot}3$	$-0{\cdot}0 \pm 1{\cdot}7$	$-0{\cdot}3 \pm 3{\cdot}2$	$-0{\cdot}6 \pm 1{\cdot}0$	$-1{\cdot}4 \pm 1{\cdot}8$	$0{\cdot}0 \pm 1{\cdot}7$	$-4{\cdot}8 \pm 5{\cdot}5$	$+1{\cdot}3 \pm 2{\cdot}7$
S. A. 3 Feb.	$-0{\cdot}5 \pm 5{\cdot}1$	$+4{\cdot}4 \pm 2{\cdot}0$	$-2{\cdot}5 \pm 4{\cdot}0$	$+0{\cdot}6 \pm 1{\cdot}3$	$+1{\cdot}1 \pm 2{\cdot}8$	$-0{\cdot}3 \pm 2{\cdot}6$	$-1{\cdot}9 \pm 3{\cdot}1$	$+3{\cdot}7 \pm 2{\cdot}0$

headed finger-eye 1 and finger-eye 2. For present purposes the scatter of the points, of which the standard deviations are a measure, is of greater significance than any bias they may show, as revealed in the means. If, for example, all the points were closely bunched but at some distance from the target, we should nevertheless have to conclude that some form of accurate positional information was available in order for the subject accurately to produce the same error at each trial. Conversely, zero bias implies nothing as to accuracy; what matters is whether the points are widely scattered.

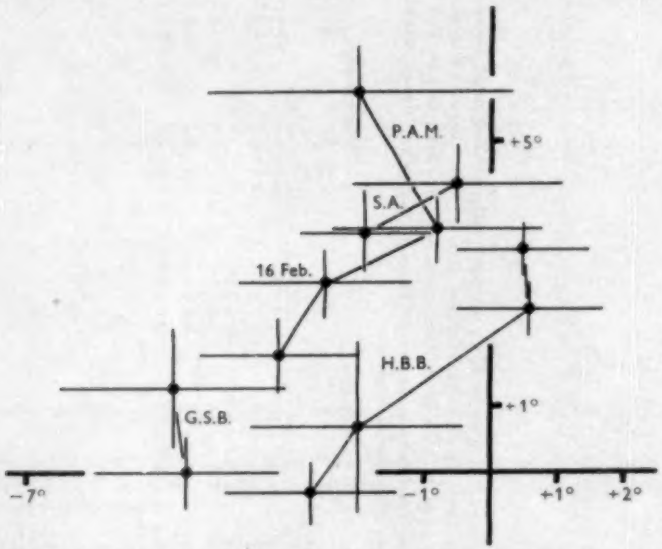

Fig. 2. The mean fixation points for all the finger-eye experiments in Table 1. The error bars show the standard errors of the means. They also indicate the relative standard deviations of individual observations; for $n = 10$ for all points except that for P.A.M. 16 February where $n = 18$. The point touched is the origin.

Bias of course would be a trivial matter could it be accounted for as an expression of the standard error of the mean, but a glance at Table 1 shows that it cannot; for the mean is frequently as much as three standard errors from the axes of co-ordinates. Similar large biases have been found in every type of experiment reported in this paper. No attempt has been made to find out what factors influence bias, but some interesting points came to light from an examination of the twelve finger-eye experiments in Table 1. The mean fixation positions with their x and y standard-error bars are plotted in Fig. 2. The most obvious thing is that x and y bias are inversely related; this is true both for the results as a whole and, more strikingly, for the individual subjects (excepting G. S. B., both of whose points, however, are of large x and small y bias). Except for H. B. B., the points for all subjects fall near a single line; for H. B. B. the line has the same slope but lies nearer the target. The direction of these lines is roughly at right angles to the direction in which the subject's right index

finger is carried to the target in the initial sensing. Thus a possible explanation is that the mean error in judging arm position is rather constant from experiment to experiment and from subject to subject in the direction of arm movement, but is much less constant at right angles. The freedom of movement of the index finger is certainly largest in this latter direction; for example, in rubbing out writing on a blackboard with the right index finger the strokes are made in the same direction as the lines on which the points lie in Fig. 2. Whether or not an explanation of this type would stand up to further investigation, it is clear that large x or large y biases are not of themselves to be taken as implying inferior performance, for the distance of the mean positions from the target is much the same for all experiments (less for H.B.B.). This fits in with the fact that a large x or y bias is not associated with a particularly large scatter of observations in that direction (indicated by the standard error bars in Fig. 2) as it is in many systems where bias is one result of poor error correction. Indeed, the only apparent trend is for x scatter to increase not with $\bar{x}$ but with $\bar{y}$. This is most conspicuous with subject P.A.M. and in him (at least) is because large positive or negative errors in x tend to be associated with large positive errors in y, as can be seen in Fig. 1. Both types of error are due to a tendency to overshoot; overshoot upwards increases the mean value of y but horizontal overshoot to left and right contributes to the standard deviation of x rather than to the mean, as further discussed on p. 566.

Results for the eye and the hand separately

At the same sessions the subjects did two other experiments designed to test the eyes and finger separately. In the first of these experiments instead of the subject's finger being placed on the flash aperture he looked at the aperture with the room lights on. He then lowered and shut his eyes, the room lights were put out, and he then, as before, opened his eyes and attempted to look at the aperture in darkness. This experiment tests only the subject's knowledge of the direction of his eyes. The results are given in Table 1 in the column headed eye-eye. In the second of these experiments his finger tip was, as before, passively placed on the flash aperture and then put down again on the table, but his task was to replace his index finger on the flash aperture. The flash aperture was a hole in a board covered in graph paper, so that the co-ordinates relative to the aperture of the estimated centre of the tip of the index finger on the board could be read off at each attempt. This experiment tests only the subject's knowledge of the position of his finger. The results appear in the column headed finger-finger.

The session began with a series of ten finger-eye observations; then followed ten eye-eye and ten finger-finger observations and finally another ten finger-eye. (In H.B.B., 4 February, finger-finger preceded eye-eye.) The results of two incomplete sessions for P.A.M., 30 January, and H.B.B., 30 January, are also given. The session P.A.M. 16 February used a different procedure designed to eliminate possible effects of progressive changes in performance during the session. Each series, finger-eye, etc., used 18 positions of the carriage given by two random sequences of the integers 1–9. Thus each position was assumed twice and twice only

in each series. Observations were made in threes, a finger-eye, an eye-eye and a finger-finger, and then a finger-eye again and so on. With this stricter technique, however, the results were not obviously different. Table 1 shows that the errors for the eye and finger separately are both substantially less than for the finger-eye series. It is of interest that the performances of the eye and the finger are roughly similar, an unforeseen finding which, however, all subsequent work has only confirmed. In so far as we are concerned with the question of the accuracy of eye movements, the eye-eye results are obviously of more significance than those of the original Sherringtonian finger-eye type of experiment, and in what follows interest is mainly centred on eye-eye experiments.

TABLE 2. The variance of finger-eye experiments in Table 1, compared with the sum of the variances of the corresponding eye-eye and finger-finger series. The figures given are the total variances for single observations, i.e. the sum of the squares of the standard deviations of x and y in Table 1. The finger-eye values are the mean of the variances of the finger-eye (1) and finger-eye (2) series, except for P.A.M. (16 February) where there is only one series

Subject and date	Eye-eye	Finger-finger	Sum of eye-eye and finger-finger	Finger-eye
P.A.M. 2 Feb.	5·2	19·7	24·9	44·2
16 Feb.	11·3	9·1	20·4	37·2
H.B.B. 4 Feb.	8·3	4·9	13·2	13·7
G.S.B. 1 Feb.	11·3	6·4	17·7	29·5
S.A. 3 Feb.	17·3	14·6	32·0	21·7
Mean vales	10·7	11·0	21·6	29·3

If the errors in the original finger-eye series were due to a combination of the errors in judging the position of the finger and the errors in judging the position of the eyes, the variance in the finger-eye series ought to be the sum, or on another view, half the sum, of the variances of the eye-eye and finger-finger series. Table 2 shows that the finger-eye variance is never less than two thirds (S.A.) of this sum and may be almost double (P.A.M.). The figures are too variable to draw firm conclusions but it seems probable that the finger-eye errors are greater than would be expected if they were a simple combination of eye and finger errors.

Variance and standard deviation are only a fair measure of accuracy of performance if the errors are normally distributed. When the x and y co-ordinates of the individual observations were plotted against the serial number of the observation no obvious regularities such as trends up or down, convergence to the true or some other value, or oscillation, could be discerned. In addition, as a test for serial correlation, the variance of readings about their mean was compared with the mean square of the difference between successive readings. If the errors are not serially correlated the ratio of these two statistics should be 1:2. The x and y

co-ordinates for each experiment in Table 2 were treated separately, giving 38 values of the ratio. The mean was 2·10, which is not significantly different from 2·00. Furthermore, the distribution of individual values around 2·00 was examined and found to be consistent with the hypothesis of no serial correlation [the largest ratio was 3·29 ($P > 0·06$), and the smallest 1·03 ($P > 0·2$)].

The same test for serial correlation has been applied to all the other experiments reported in this paper, with similarly negative results. The figures are only given, however, for the experiments of Table 6.

Possible reasons for the large errors found

The scatter shown even in the eye-eye experiments was unexpectedly large and most of the subjects were surprised at their own inaccuracy. It is difficult to believe that if Sherrington had obtained similar results he would have called them 'good' or felt that they proved that the eyes possessed accurate position sense. Several possible sources of error in the present experiments that might account for this apparent discrepancy were considered and eventually rejected. In Sherrington's experiments the fixation mark was free to move vertically as well as horizontally, but it is extremely difficult to see why restricting the degrees of freedom to one in the present experiments could make the performance worse—it ought to make it better; indeed, as will be seen shortly, when the aperture did not move at all the errors were much diminished in the horizontal direction. Sherrington anaesthetized the front of the eyeballs with cocaine, but this again would be expected, if anything, to make matters worse rather than better. To make sure, both eyes were thoroughly anaesthetized by dropping 4% cocaine into the conjunctival sacs and repeating this three times at intervals of about 3 min; a series of 10 eye-eye observations was then made in the usual way. The mean errors and the standard deviations of single observations were $\bar{x}$, $-1·4° \pm 2·6°$; $\bar{y}$, $+0·5° \pm 0·9°$. Total variance 7·5. These values are quite unexceptional, as may be seen from Tables 1 and 2. Another possibility is that persisting after-images cause the subject unwittingly to deviate his gaze. In finger-eye and eye-eye experiments each observation takes about 1 min to make. If the subject does not pause between observations, positive after-images from previous attempts are feebly visible in the dark and might influence him. There are also faint after-images of objects in the room. Experiments were therefore done in which the subject sat in darkness before making an observation, to allow after-images to fade away. These observations were made alternately with observations at the usual interval in order to obtain a strict comparison. Preliminary trials showed that the after-image of a flash did not remain visible in darkness for longer than about a minute, but, to

allow a margin, a period of 2 min was adopted. To avoid fresh after-images of the room and equipment the flash aperture was not illuminated by turning on the room lights, but a small faint fixation light was provided just above the aperture. At the end of 2 min in the dark the subject bit the dental grip and gazed at the fixation light; this was then put out and the ordinary procedure for an eye-eye observation followed. Immediately the results of this observation had been written down another was made, with an after-image of the flash visible; then followed another 2 min in darkness, and so on. Two series each of nine pairs of observations were made. In the first (series *A*) the dim fixation light without room lighting was used throughout; in this series after-images of objects in the room were faint or absent, as they faded more quickly than the flash after-image. In series *B* bright room lighting was used to illuminate the aperture

TABLE 3. The errors made in eye-eye observations after 2 min in darkness compared with observations (made alternately) directly after putting out the room lights. Each mean is from nine observations in which the carriage assumed each of the nine possible positions in random order. Subject P. A. M. Means and standard deviations of single observations, in degrees

	Series *A*		Series *B*	
	2 min in dark	Immediate	2 min in dark	Immediate
$\bar{x}$	$-0{\cdot}6 \pm 1{\cdot}8$	$-0{\cdot}1 \pm 1{\cdot}9$	$0{\cdot}0 \pm 1{\cdot}8$	$-0{\cdot}6 \pm 1{\cdot}1$
$\bar{y}$	$+1{\cdot}9 \pm 0{\cdot}6$	$+1{\cdot}6 \pm 0{\cdot}7$	$+1{\cdot}7 \pm 0{\cdot}8$	$-0{\cdot}2 \pm 0{\cdot}8$

in those observations which did not follow a period in darkness; in these observations after-images both of room objects and of the previous flash could be seen; they were thus strictly comparable with the previous eye-eye experiments. The results are given in Table 3; they show that the presence of after-images does not make the subject less accurate. When using the fixation light for both observations of a pair there is no perceptible difference in performance (Series *A*). When the room lights were used for the second of the pair (Series *B*) the errors were, if anything, diminished.

A final possibility is that Sherrington's subjects were better trained. No experiments specifically to investigate training have been done, but the results shown in Table 1 lend no support to the idea that training would have a very large effect. Subjects H.B.B., S.A. and G.S.B. had no previous experience of this type of experiment and before starting on the series shown in Table 1 they only had two or three practice trials. The author, P.A.M., however, had been doing very similar experiments for several days before the Table 1 results were obtained. Nevertheless he was, by and large, worse than the others. Only P.A.M. and H.B.B. did experiments on more than one day. P.A.M. was, if anything, rather worse on 2 and 16 February than he had been on 30 January, but H.B.B. improved at his second session. As regards improvement with practice

during a single session, there were five occasions on which finger-eye runs were repeated at the end of a session. Three of these showed greater inaccuracy than in the first run; two showed less. Only H. B. B. did two such sessions; he deteriorated during the first and improved during the second. Table 1 therefore reveals no clear tendency to improve with training, either over several days or during a single session. Some other experiments, described below (Table 6), did show a possible training effect, but it was not large, amounting to a reduction in standard deviation of about 50%.

There seems no reason, therefore, to doubt that the power of directing the eyes in darkness in randomly selected directions is as poor as the results in Table 1 suggest. Sherrington's type of experiment, which tests the power to look towards a point previously located by the hand, gives results considerably worse than that in which the point has first been located by looking at it. It will now be shown that even an eye-eye experiment somewhat exaggerates the inaccuracy of the eye if the results are presented as they are in Table 1.

Results for different positions of the object considered separately

It was early noticed that the errors tended to be larger when the carriage was deviated widely from the centre position and that the usual error at these times was an overshoot, the eye deviating too far. An eye-eye experiment was therefore carried out in which a sufficiently large number of observations was made for the results at different carriage positions to be treated separately. Only five positions were used (instead of the usual nine), 20° L, 10° L, centre, 10° R and 20° R. Observations were made in sets of these five positions taken in random order, each set having

TABLE 4. The errors made in eye-eye observations at five different positions of the flash aperture relative to the subject (P. A. M.). Twenty observations at each position. Errors to the left −, to the right +. Mean errors and standard deviations of single observations in degrees

	20° to the left	10° to the left	Straight ahead	10° to the right	20° to the right
$\bar{x}$	$-1{\cdot}6 \pm 2{\cdot}0$	$-2{\cdot}8 \pm 1{\cdot}3$	$-1{\cdot}0 \pm 1{\cdot}0$	$+0{\cdot}6 \pm 1{\cdot}2$	$+0{\cdot}9 \pm 1{\cdot}4$
$\bar{y}$	$+0{\cdot}3 \pm 0{\cdot}9$	$+0{\cdot}6 \pm 0{\cdot}8$	$+0{\cdot}7 \pm 0{\cdot}9$	$+1{\cdot}0 \pm 0{\cdot}9$	$+1{\cdot}2 \pm 1{\cdot}2$

a different random order, until twenty sets had been done. The results are given in Table 4. The values for $\bar{x}$ show the expected overshoot. The horizontal scatter is least in the centre (S.D. $\pm 1{\cdot}0$) and increases with deviation (S.D. up to $\pm 2{\cdot}0$ and $\pm 1{\cdot}4$). In the vertical direction the bias changes slightly (the subject tends to look higher as he looks less to the left, or more to the right) but the scatter shows little change. When the observations at the different positions were pooled and the grand means and standard deviations taken in exactly the same way as in Table 1,

the results obtained were $\bar{x}$, $-0{\cdot}8° \pm 2{\cdot}0°$; $\bar{y}$, $+0{\cdot}8° \pm 1{\cdot}0°$, values similar to those in the eye-eye column of Table 1. The horizontal standard deviation obtained in this way is larger than all except one of the separate standard deviations in Table 4, because the variable x overshoots in Table 4 contribute to the pooled standard deviation but not, of course, to the separate ones. As we are interested chiefly in scatter and not in bias it is clear that the values for standard deviation given in Table 4 are more relevant than the larger ones obtained on the pooled readings or in Table 1. Table 4 shows that when the eyes are looking straight ahead, or are deviated up to some 10°, the scatter in both horizontal and vertical directions reduces to a standard deviation of roughly $\pm 1°$. This is probably a good estimate of the true accuracy of directing the eyes in the dark. In various other situations about to be described, which were tried in case they revealed a greater accuracy, nothing substantially better was achieved.

The ability to fixate an object held in the fingers

In the finger-eye experiments already described there is a delay of several seconds between locating the object by touch and directing the eyes to it, which is obviously prejudicial to accuracy. In an experiment which avoided this delay the flash aperture was not a hole in a board, as previously, but a window at the end of a metal tube 1 cm in diameter pointing at the subject's face. The subject grasped the end of the tube between thumb and forefinger and himself swung the carriage to left or right with this hand. With the carriage in the starting position the subject fixated the window with the room lights on. They were then extinguished; the subject moved the carriage through some angle of roughly 10–20°, meanwhile attempting to maintain fixation, and, with the carriage again stationary, pressed the button to fire the flash with his other hand. (The exact angle through which the carriage moved was not recorded.) The subject first made ten observations with the carriage initially straight ahead and then swung to the left; then ten after swinging to the right. Next, the observations were made with the initial position approximately 15° to the left and the carriage swung towards the centre, and this was repeated with the initial position 15° to the right. This experiment tests a type of finger-eye co-ordination that is likely to be biologically more useful and is also more familiar to subjects than the somewhat artificial task in Sherrington's original procedure; but it is not much better performed, as the results, given in Table 5, show. Most of the results show a mean overshoot which, while it is larger when the demanded eye movement is away from the centre, is clearly not just a characteristic of movements away from the primary position (a similar overshoot will be found in the

experiments of Table 11). The standard deviations do not vary greatly around an average value of $\pm 1{\cdot}5°$. Both subjects were left-handed and the scatter is somewhat smaller when the left hand was used.

The experiments which start with the carriage in the centre are roughly comparable with the finger-eye experiments of Table 1, if the ten observations with movement to the left are pooled with those to the right. The means and standard deviations of the pooled series are also given in Table 5.

TABLE 5. The errors made in attempting to look at the flash aperture held between thumb and forefinger. For each series of ten observations is given the mean horizontal error, − to the left, + to the right, and the standard deviation of single observations about this mean. Figures are also given for the first two series of each session pooled and considered as a single experiment. All values in degrees

Subject, date and hand used	Aperture initially central		Aperture swung towards centre	
	Swung left	Swung right	From the left	From the right
T.D. 31 Dec. Left hand	$-3{\cdot}0 \pm 1{\cdot}1$	$+2{\cdot}7 \pm 1{\cdot}5$	$-1{\cdot}6 \pm 1{\cdot}5$	$+0{\cdot}3 \pm 1{\cdot}1$
	$-0{\cdot}1 \pm 3{\cdot}2$			
T.D. 2 Jan. Right hand	$-1{\cdot}4 \pm 1{\cdot}8$	$-0{\cdot}4 \pm 1{\cdot}5$	$+0{\cdot}4 \pm 2{\cdot}0$	$+1{\cdot}8 \pm 1{\cdot}3$
	$-0{\cdot}9 \pm 1{\cdot}7$			
P.A.M. 1 Jan. Left hand	$-3{\cdot}4 \pm 1{\cdot}6$	$+2{\cdot}6 \pm 1{\cdot}3$	$-2{\cdot}7 \pm 1{\cdot}9$	$-0{\cdot}3 \pm 1{\cdot}3$
	$-0{\cdot}4 \pm 3{\cdot}4$			
P.A.M. 2 Jan. Right hand	$-2{\cdot}6 \pm 1{\cdot}2$	$+3{\cdot}0 \pm 1{\cdot}9$	$-1{\cdot}5 \pm 1{\cdot}3$	$+2{\cdot}4 \pm 2{\cdot}2$
	$+0{\cdot}2 \pm 3{\cdot}3$			

As in the previous experiment, and for the same reason, the resultant standard deviation is larger than the separate values. The pooled standard deviations are still, however, rather less than those for finger-eye runs in Table 1, possibly, as suggested, because there is no delay here between touching the aperture and attempting to look at it. It should also be mentioned that in the present experiment the finger and thumb holding the metal tube rubbed it with a pin-rolling motion. This was because Helmholtz (1867, p. 613; 1925, p. 260) states that when he attempted to look at his index finger with his eyes closed he found that, on opening them, convergence was more accurate if he rubbed the tip of his index finger with his thumb. A similar movement was made here in case it improved accuracy in this type of task too, but no controls were done to see whether it did.

Eye and finger observations with the object straight ahead

Both the last experiments suggest that when overshoot is discounted the true scatter of eye positions is about $\pm 1°$. It was of interest to see how much this figure could be improved on when the task was simplified by keeping the target stationary in the central position. Series of finger-finger observations were run for comparison. In addition, combined eye-eye

and finger-finger experiments, in which the subject attempted simultaneously to touch and look at the target, were made to see how the hand and eye interacted. The procedure and equipment were the same as for the experiments of Table 1 with minor changes. The subject was the author; only the left (dominant) eye was used, the other being covered with a shade; the left arm was used instead of the right (he is left-handed) and was moved actively not passively; the subject did not point with his index finger but held a mapping pin between index finger and thumb, and stuck it into the board. In a combined observation the subject put the pin through the centre of the flash aperture (diameter 3 mm) and, while holding it, looked at the head of the pin. He then shut his eyes and looked down, removed the pin, rested his arm on the table and put the light out. He now opened his eyes and attempted to replace the pin in the aperture (but almost invariably stuck it into the board). At the same time he attempted to look at the aperture too, and, immediately the pin was firmly in, he moved his hand to the left and pressed the flash button. (Ideally the flash should have been fired at the same moment that the pin made contact, but this was unsatisfactory as the hand was often in front of the aperture.) In an eye-eye observation he looked at the aperture, shut his eyes and looked down, put out the light, attempted to look at the aperture and pressed the button. In a finger-finger observation he put the pin in the aperture, shut his eyes, lowered his arm to the table again, put out the light, opened his eyes and tried to put the pin back. The results are given in Table 6, which shows the means and standard deviations for x and y separately, and in Table 7 where the sum of the x and y variances appears. The figures at the right-hand end of both tables show that in separate eye-eye and finger-finger experiments the eye and the hand are very similar in performance. The errors in the eye-eye series are, at best, slightly less than those for central carriage positions in Table 4. (In fact the difference is even less than appears, for the figures in Table 4 are from runs of 20 observations. If, to be strictly comparable, each is recomputed as two series of 10, the scatter of the variances widens and two series show total variances of only 1·3.) The figures for the combined observations appear at the left of Tables 6 and 7. The eye errors are regularly larger than in the immediately preceding or succeeding separate series, but this might be because the subject is distracted by moving his hand away from the target before pressing the flash button. No explanation can be offered, however, for the interesting finding that the finger errors are substantially smaller in the combined observations. The combined observations were also worked out to give the mean, variance and standard deviation of eye position relative to finger position (instead of relative to target). The figures appear in the central columns

TABLE 6. The errors in eye-eye and finger-finger observations made simultaneously and afterwards separately with a fixed target. Ten trials in each series. Subject P.A.M. The numbers show the order in which the experiments were done. On 23 January series 1 and 2 were immediately consecutive and so were 3 and 4, and 5 and 6. Otherwise about 3 min intervened between series on each day, except for a break of 10 min between series 6 and 7 on 24 January. The central column gives the mean distances between the finger position and the eye position in the combined experiments and the standard deviations of single measurements. Elsewhere the figures are, as usual, for the mean errors measured from the target and the standard deviations of single observations. All figures in degrees

		Simultaneous							Separate					
		Eye-eye		Finger-finger		Eye from finger			Eye-eye alone			Finger-finger alone		
		$\bar{x}$	$\bar{y}$	$\bar{x}$	$\bar{y}$	$\bar{x}$	$\bar{y}$		$\bar{x}$	$\bar{y}$		$\bar{x}$	$\bar{y}$
23 Jan.	(1)	$+2{\cdot}0\pm0{\cdot}9$	$+0{\cdot}2\pm0{\cdot}3$	$+1{\cdot}8\pm0{\cdot}4$	$+0{\cdot}9\pm0{\cdot}9$	$+0{\cdot}2\pm1{\cdot}0$	$-0{\cdot}7\pm0{\cdot}9$	(3)	$+0{\cdot}1\pm1{\cdot}7$	$-0{\cdot}6\pm0{\cdot}9$	(5)	$+4{\cdot}2\pm1{\cdot}4$	$+1{\cdot}3\pm0{\cdot}6$
	(2)	$0{\cdot}0\pm1{\cdot}7$	$-1{\cdot}2\pm1{\cdot}5$	$+1{\cdot}6\pm0{\cdot}6$	$+1{\cdot}7\pm0{\cdot}9$	$-1{\cdot}7\pm2{\cdot}1$	$-2{\cdot}9\pm1{\cdot}9$	(4)	$+0{\cdot}3\pm0{\cdot}9$	$-0{\cdot}8\pm0{\cdot}6$	(6)	$+2{\cdot}4\pm1{\cdot}5$	$+0{\cdot}9\pm0{\cdot}9$
24 Jan.	(1)	$+1{\cdot}2\pm1{\cdot}5$	$-3{\cdot}2\pm1{\cdot}2$	$+0{\cdot}6\pm0{\cdot}4$	$+1{\cdot}0\pm0{\cdot}7$	$+0{\cdot}6\pm1{\cdot}4$	$-4{\cdot}2\pm1{\cdot}5$	(2)	$+0{\cdot}9\pm0{\cdot}9$	$-0{\cdot}2\pm0{\cdot}5$	(3)	$+2{\cdot}8\pm0{\cdot}7$	$+0{\cdot}7\pm0{\cdot}7$
	(4)	$+0{\cdot}6\pm0{\cdot}9$	$-0{\cdot}6\pm0{\cdot}6$	$+0{\cdot}8\pm0{\cdot}6$	$+0{\cdot}7\pm0{\cdot}7$	$-0{\cdot}2\pm1{\cdot}0$	$-1{\cdot}3\pm0{\cdot}6$	(5)	$+1{\cdot}1\pm0{\cdot}7$	$0{\cdot}0\pm0{\cdot}5$	(6)	$+2{\cdot}6\pm0{\cdot}7$	$+1{\cdot}2\pm0{\cdot}6$
	(7)	$0{\cdot}0\pm1{\cdot}0$	$-0{\cdot}4\pm1{\cdot}1$	$+1{\cdot}1\pm0{\cdot}6$	$+2{\cdot}1\pm0{\cdot}7$	$-1{\cdot}2\pm1{\cdot}2$	$-2{\cdot}5\pm1{\cdot}0$	(8)	$+0{\cdot}1\pm0{\cdot}8$	$-0{\cdot}3\pm0{\cdot}8$	(9)	$+0{\cdot}9\pm0{\cdot}8$	$+1{\cdot}0\pm1{\cdot}0$

TABLE 7. The variances of the eye-eye and finger-finger experiments of Table 6. The figures given are the total variances for single observations, i.e. the sum of the squares of the standerd deviations of x and y in Table 6

		Simultaneous					Separate		
Date		Eye-eye variance	Finger-finger variance	Their sum	Eye from finger variance		Eye-eye variance		Finger-finger variance
23 Jan.	(1)	0·9	1·0	2·0	2·0	(3)	3·8	(5)	2·4
	(2)	5·2	1·2	6·4	7·9	(4)	1·1	(6)	3·0
24 Jan.	(1)	3·9	0·6	4·6	4·4	(2)	1·0	(3)	1·0
	(4)	1·3	0·8	2·1	1·3	(5)	0·8	(6)	0·9
	(7)	2·4	1·0	3·3	2·3	(8)	1·3	(9)	1·6

of Tables 6 and 7. If there is no interaction between hand and eye the total variance of eye on finger should equal the sum of the variances of eye on target and finger on target. Table 7 shows that this seems to be roughly true on 23 January, but on 24 January (when the subject's performance is less erratic and on the whole better) the eye-on-finger variance is significantly less than the sum of the other two, being, in fact, roughly equal to the eye-on-target variance. It cannot be said, therefore, that in a combined observation the subject gives up trying to look at the target and tries only to look at his finger, but there are times when he apparently divides his attention roughly equally between the two.

All the x and y series in Table 6 were tested for serial correlation by comparing the variance with the mean square of the differences between successive observations. The 40 ratios resulting had a mean of 2·04, which is not significantly different from 2·00. The highest ratio was 3·33 ($P > 0{\cdot}05$), the lowest 0·73 ($P > 0{\cdot}06$) and the distribution of the other values was likewise satisfactory.

The ability to maintain fixation in the dark. Alterations in convergence

A final simplification of the original task was to test the power of merely continuing to look in the same direction in the dark. The subject gazed at a dim fixation light immediately above the flash aperture, the room being otherwise dark. When he was ready he threw a switch which extinguished the fixation light and started an automatic device which fired the flash after a pre-set delay. In the darkness he attempted to maintain fixation on the light. Both eyes were used and the head was steadied as usual by biting a dental grip. The early results showed that with the fixation light at 57 cm the first thing that happened was that the eyes diverged. When, however, prisms and lenses were put in front of the eyes, so chosen that the fixation light appeared to be at infinity, then the eyes tended to converge in darkness. Trial and error showed that for the author little change in convergence resulted when the apparent distance was 150 cm. (With a real distance of 57 cm this necessitated +1 dioptre in front of each eye and a total of 7 prism dioptres, base inwards. One prism dioptre deviates light from an object ahead through 1/100 radian. The author is emmetropic and tested with a Maddox rod has no latent squint at 6 m.) To show the effect of distance clearly, Table 8 compares the results obtained at 150 cm with those for an apparent distance of 25 cm (−2·25 dioptres in front of each eye and a total of 14 prism dioptres, base outwards). The delay before the flash was fired was alternately 1·0 and 5·0 sec. Thus any progressive changes in performance should affect the results at the two intervals equally. In each observation the position of the after-image for each eye was recorded separately. Subsequently

the mean position and the convergence or divergence were worked out from these figures. The vertical co-ordinates were not recorded. A separate experiment showed that no corrections for magnification were required. The results show that the scatter of the mean fixation points is much the same at the two distances, but that the eyes rapidly diverge when the fixation point is close. At 5·0 sec the mean convergence remaining is appropriate to an object at a distance of 37 cm.

TABLE 8. The errors made in attempting to maintain fixation in the dark. Flash gun fired after either 1·0 or 5·0 sec. Subject P. A. M. For each series of ten observations are given the mean horizontal error of the mid position of the fixation points, in degrees, for the two eyes and the standard deviation of single observations; and the mean alteration in convergence, in degrees, and the standard deviation of single observations. Increased convergence +, divergence −

	Apparent distance of object 25 cm		Apparent distance of object 150 cm	
	1·0 sec	5·0 sec	1·0 sec	5·0 sec
Errors of mid position	$+0·3 \pm 0·3$	$+0·4 \pm 0·6$	$+0·2 \pm 0·1$	$+0·2 \pm 0·4$
Change of convergence	$-1·0 \pm 0·5$	$-4·7 \pm 1·7$	$+0·5 \pm 0·2$	$+0·5 \pm 0·6$

TABLE 9. Similar experiments to Table 8 with subject T. D. and different times and distances. All figures in degrees

	1·3 sec	3·6 sec	7·0 sec	9·0 sec
Object at 44 cm				
Error of mid position	$+0·1 \pm 0·3$	$-0·2 \pm 0·7$	$0·0 \pm 0·9$	—
Change of convergence	$-0·8 \pm 0·1$	$-2·1 \pm 0·5$	$-2·9 \pm 0·8$	—
Object at 150 cm				
Error of mid position	$0·0 \pm 0·1$	$-0·2 \pm 0·6$	$-0·2 \pm 1·2$	$-0·5 \pm 1·5$
Change of convergence	$-0·2 \pm 0·1$	$-0·2 \pm 0·2$	$0·0 \pm 0·1$	$0·0 \pm 0·3$

Table 9 gives results from another subject for distances of 44 and 150 cm. In these experiments a series of ten observations was made at 1·3 sec delay and then a series at 3·6 sec and so on. Hence the results cannot be guaranteed free from the effects of progressive change. At 150 cm there is a steady increase with time in the scatter of the mean fixation points without any significant alteration in convergence. At 44 cm the figures for the mean fixation points are very similar, but there is also a large divergence. At 7·0 sec the mean convergence remaining is appropriate to an object at 68 cm.

The fact that two subjects showed little change in convergence in darkness after fixating an object at 150 cm suggests that in the absence of any stimulus to convergence from the presence, or recent presence, of a fixation object the optic axes would tend naturally to assume this degree of convergence. It is of interest to recall that Rayleigh (1883) found he became short-sighted in an almost dark room and Campbell's and others' measurements show the average refractive power of the lens in the dark to be some 0·6 dioptres (Campbell, 1954). Thus it seems probable that in the dark the eyes are both focused and converged for objects at about 150 cm, 'night myopia' being accompanied by 'night squint'. Whiteside

(1952) showed that changes in accommodation similar to those in the dark occur when the eye is presented with a uniform bright field devoid of detail to fixate; it would be interesting to know if the same is true for convergence.

The above results strongly suggest that convergence and mean direction are independently determined. Several experiments were done with other apparent distances of the object which did not show the dissociation as clearly as those given in Tables 8 and 9, but none were inconsistent with this conclusion. The scatter of the mean fixation points at 5·0 sec and particularly at 7·0 sec is much the same as the scatter in the experiments of Table 6. These times are similar to the times taken to make an eye-eye (or a finger-eye) trial. Thus the accuracy of merely attempting to maintain a fixed direction in the dark is not clearly better than the accuracy of looking away and attempting to refixate. On the other hand, when the object is close the eyes rapidly diverge in the dark, and the amount of this divergence shows a large scatter.

It is relevant to the question of conscious position sense in the eyes that subjects are quite unaware of these alterations of convergence in the dark. With close introspection they are sometimes vaguely conscious of saccadic changes of mean fixation point, but this is very likely because rapid movements can be detected by sense organs in the eye lids.

The ability to hold the hand still in the dark

The author made comparable experiments on the steadiness of the hand. A mapping pin was stuck into a hole in the board 57 cm in front of him. To alter conditions as little as possible the dental grip was used. The subject took hold of the pin with his right hand and shut his eyes. He then withdrew it, held it as steady as possible just clear of the board for either 1·0 or 5·0 sec, and then attempted to replace it in the hole. Meanwhile he kept his eyes shut, of course. The timing was done by listening to the clicking of the relay in the automatic device previously used for firing the flash. The 1·0 and 5·0 sec delays were used alternately, the experiment consisting of ten such pairs. The results are given in Table 10. Once again it proves that the errors in the hand are remarkably similar to those in the eyes under similar conditions.

Looking from one fixation point to another

As a quite different task, experiments were done to see how accurately it was possible to swing the eye from one fixation point to another in the dark. For this purpose two dim fixation lights subtending 25° at the subject's eye were arranged. Below one of them, say the right-hand one, was the flash aperture. Only one eye, the subject's dominant eye, was used, the other being covered with a shade. With the room lights out the

subject practised looking from one light to the other. Finally, he fixated the left-hand light and threw a switch which extinguished both. He immediately attempted to fixate the right-hand light and as soon as he was satisfied (that is to say, within a second or two) he pressed a button to fire the flash. Only errors in the horizontal direction were recorded. Two subjects were used; both showed a marked tendency to overshoot by one or two degrees and a standard deviation of single observations about the mean of about one degree. Table 11 shows that (as in the experiments of Table 5) the overshoot was present whether the final fixation point was

TABLE 10. The errors made in attempting to hold the hand steady. Board 57 cm from face. Pin withdrawn from board and replaced after either 1·0 or 5·0 sec. Subject P.A.M. For each series of ten observations are given the mean errors and the standard deviations of single observations in degrees

	1·0 sec	5·0 sec
$\bar{x}$	$+0{\cdot}1 \pm 0{\cdot}2$	$+0{\cdot}2 \pm 0{\cdot}4$
$\bar{y}$	$-0{\cdot}2 \pm 0{\cdot}1$	$-0{\cdot}1 \pm 0{\cdot}2$

TABLE 11. The errors made in attempting to move the eye in darkness between two fixation points subtending an angle of 25° at the eye. On each day experiments succeeded each other without a break in the numbered order. The positions of the first and second fixation points relative to the subject are given by the letters L, C and R, L being 25° to the left of the primary (straight ahead) position, C the primary position and R 25° to the right of the primary position. The figures given are the means of the actual movements and the standard deviations of single observations about these means. Thus 26·4 is a mean overshoot of 1·4°. All values in degrees

P.A.M.	20 Oct.	(1) L to C ($n = 20$), $26{\cdot}4 \pm 1{\cdot}0$	(2) R to C ($n = 20$), $25{\cdot}7 \pm 1{\cdot}1$
	22 Oct.	(1) C to L ($n = 10$), $26{\cdot}6 \pm 0{\cdot}8$	(2) R to C ($n = 10$), $25{\cdot}5 \pm 1{\cdot}5$
		(3) C to L ($n = 10$), $27{\cdot}0 \pm 0{\cdot}8$	(4) R to C ($n = 10$), $26{\cdot}0 \pm 1{\cdot}2$
	8 Feb.	(1) L to C ($n = 10$), $27{\cdot}5 \pm 1{\cdot}0$	(2) C to R ($n = 10$), $27{\cdot}6 \pm 1{\cdot}3$
T.D.	22 Oct.	(1) R to C ($n = 20$), $25{\cdot}7 \pm 0{\cdot}9$	(2) C to L ($n = 20$), $26{\cdot}1 \pm 1{\cdot}2$

in front of the subject or to one side or the other, and whether the eye movement was to the left or the right. The results, as a whole, show that moving the eyes from one fixation point to another is no more accurately performed than the other tasks already tested.

Repositioning a luminous point in the dark

A last experiment explored the possibility that the eye muscles might give accurate information about eye position which was available for judging the position of external objects, but in some way could not be used for directing the eyes accurately in the dark. The subject was shown a dim light in front of him in an otherwise dark room. It was then extinguished by an assistant, moved horizontally either to left or right for several degrees and put on again. The subject then gave the assistant instructions to move it until he was satisfied that it had returned to the

original position. This operation took between 5 and 10 sec. Alternating with these observations ordinary eye-eye observations were made. The subject looked at the dim light, it was put out, he looked away and then attempted to look at it again; after 5 sec the flash immediately below the light was fired. Only horizontal errors were recorded. Ten such pairs were carried out. In the repositioning series the mean error and the standard deviation of single observations about the mean was $-0{\cdot}6° \pm 0{\cdot}7°$ and in the eye-eye series $-0{\cdot}1° \pm 0{\cdot}6°$. Similar results were obtained in a nearly identical experiment on another subject. It is clear, therefore, that there is nothing exceptionally accurate about repositioning.

In preliminary trials before the above series were begun an unexpected result occurred. Owing to a misunderstanding the assistant moved the light in the opposite direction to that asked for by the subject. He also moved it rather slowly, at a rate of perhaps half a degree a second, and kept stopping. In one trial the assistant moved the light some 2° to the subject's left while it was out; when he put it on he moved it still further to the left, the subject having asked for it to be moved to the right. Nevertheless when, after several small movements, it reached 4·7° to the left the subject declared himself satisfied. In the next trial the initial movement was to the right and the final position, which was fully accepted by the subject, was 9·7° to the right. This aroused suspicion and the misunderstanding came to light. Subsequently the assistant moved the light more rapidly and in the correct direction, but the above experience shows how very uncertain a subject is of his eye position if it changes slowly. The phenomenon of autokinesis shows that he may be equally uncertain when his eyes do not move at all.

DISCUSSION

The first series of experiments show that errors of the order of 5° are made in attempting to look, in the dark, at a point previously located by touching it with a finger. As already said it is difficult to believe that if Sherrington had obtained similar results he would have spoken of them as he did. What he wrote implies that in his experiments the subject was able to direct his eyes so that when the light came on the object was at the fixation point; that at any rate is the most likely interpretation of his phrase 'remains good' in the quotation given in the introduction. Remains good by comparison with what? Only it would seem by comparison with the power to direct the gaze with the light on, and with the light on the object can, of course, be precisely fixated. This interpretation is supported by an otherwise obscure sentence in the 1918 paper. Speaking of the experiments in a dark room, with the surface of the eyes cocainized,

Sherrington says: 'The direction of the eyeball is then judged almost as accurately as with the use of the retina in the undarkened room'. This only makes sense if the expression 'judging the direction of the eyeball with the use of the retina' is intended to refer to the act of fixation. The whole sentence would take on the proposed meaning unambiguously if the word 'judged' were altered to 'adjusted'. It must be admitted, however, that if this interpretation is correct it implies that Sherrington either accepted or overlooked the improbable consequence that position sense in the arm must be equally accurate, for in his experiments the position of the mark was only known through the arm.

As it is not known how Sherrington determined the direction of the eyes when the light was switched on, it is impossible to account with certainty for the presumed differences between his results and mine. Although it seems most unlikely, attention should perhaps be drawn to the possibility that Sherrington relied on his subjects' statements of where they were looking when the lights came up. Under such conditions it might be difficult to avoid unconsciously fixating the mark as soon as it could be seen and thereby achieving a spurious accuracy. The only thing that makes this suggestion at all plausible is that it is difficult to think of any better method that could have been employed instead, within the framework of Sherrington's published account.

The type of experiment that Sherrington described does not reveal the best performance of which the eye is capable. When the object is located by first fixating it in the light, and when only one direction of object is considered at a time, the scatter is reduced to a standard deviation about the mean position of about $\pm 1°$. Even this accuracy will seldom bring the object on to the fixation area of the fovea, for Barlow (1952) has shown that when refixating a visible object successive fixation points are scattered with a standard deviation of only 5′ of arc. The performance in the dark would, therefore, have to be improved by a factor of 12 to match the performance in the light; and this does not take into account any mean bias that is introduced in the dark.

An unforeseen finding was the close similarity in the accuracy of the eyes and the hand. Perhaps it ought to have been foreseen, for joint use of the eye and hand is a feature of human life and it is not unlikely that for this purpose the most economical arrangement would be to match their performances. When, for instance, the fingers make contact with an object which arouses interest, there is no need for the mechanism which turns the eyes towards it to be able to work to closer limits than the sensory receptors in the arm that report its position in the first place.

Whether the accuracy of movement found in this paper is held to require positional information from eye-muscle receptors is a matter of opinion.

If it is required, no very large number of receptors would seem to be needed to supply it. Certainly the present experiments do not suggest that the type of task investigated here is one of those that the extremely numerous sense organs in eye muscles are employed to optimize.

SUMMARY

1. Eye positions in darkness were measured by an after-image technique.

2. When attempts were made, in the dark, to look towards an object previously located by fixating it with the lights on, errors were made such that the standard deviation of successive attempts around their mean position was roughly $\pm 1°$ in both horizontal and vertical directions.

3. Errors similar in magnitude were made when pointing with the forefinger in the dark at an object previously located by touching it with that finger.

4. Larger errors were made if the object moved between attempts or if the subject had to look towards an object located by touch. When the object occupied randomly selected positions lying between 20° to the left and 20° to the right of the subject and the task was to look towards the object in the dark after locating it by touch, the errors in the horizontal direction showed a standard deviation of about $\pm 5°$.

5. The ability merely to maintain fixation when the room was put in darkness was roughly the same as the ability to hold the hand steady at arm's length. After 5 sec both showed a standard deviation in the horizontal direction of about $\pm \frac{1}{2}°$.

6. When using both eyes, changes of convergence of several degrees might occur in the first few seconds of darkness. These changes proved to be independent of the above changes in fixation point (which refer to the mid position of the two fixation points when both eyes were used).

7. A general finding of interest was the closely similar accuracy in directing the eyes and the hand in the dark.

My warmest thanks are due to Mr T. Davies who made much of the apparatus and performed several of the experiments.

Note added in proof. In the present paper and in that of Brindley & Merton (1960) the work of Irvine & Ludvigh (1936) and Ludvigh (1952*a*, *b*), which had led them to similar conclusions, was regrettably overlooked.

Irvine, S. R. & Ludvigh, E. (1936). Is ocular proprioceptive sense concerned in vision? *Arch. Ophthal., N.Y.*, **15**, 1037–1049.

Ludvigh, E. (1951*a*). Possible role of proprioception in the extra-ocular muscles. *Arch. Ophthal.* **48**, 436–441.

Ludvigh, E. (1952*b*). Control of ocular movements and visual interpretation of environment. *Arch. Ophthal., N.Y.*, **48**, 442–448.

REFERENCES

BARLOW, H. B. (1952). Eye movements during fixation. *J. Physiol.* **116**, 290–306.

BRINDLEY, G. S. & MERTON, P. A. (1960). The absence of position sense in the human eye. *J. Physiol.* **153**, 127–130.

CAMPBELL, F. W. (1954). The minimum quantity of light required to elicit the accommodation reflex in man. *J. Physiol.* **123**, 357–366.

FICK, E. (1905). Die Verlegung der Netzhautbilder nach aussen. *Z. Psychol. Physiol. Sinnesorg.* **39**, 102–115.

HELMHOLTZ, H. (1867). *Handbuch der physiologischen Optik*, 1st ed. Leipzig: Voss. English translation by SOUTHALL, J. P. C. (1925), *Helmholtz's treatise on physiological optics*, vol. 3. Menasha, Wisconsin: Optical Society of America.

RAYLEIGH, LORD (1883). On the invisibility of small objects in a bad light. *Proc. Camb. phil. Soc.* **4**, 324. Reprinted in RAYLEIGH, LORD (1900). *Scientific Papers*, vol. 2, p. 187. Cambridge University Press.

RIVERS, W. H. R. (1900). Vision. In SCHÄFER, E. A., *Textbook of Physiology*, p. 1134. Edinburgh and London: Pentland.

SANDSTRÖM, C. I. (1951). *Orientation in the present space*. Stockholm: Almquist and Wiksell.

SHERRINGTON, C. S. (1897). Further note on the sensory nerves of muscles. *Proc. Roy. Soc.* **61**, 247–249.

SHERRINGTON, C. S. (1898). Further note on the sensory nerves of the eye muscles. *Proc. Roy. Soc.* **64**, 120–121.

SHERRINGTON, C. S. (1918). Observations on the sensual rôle of the proprioceptive nerve-supply of the extrinsic ocular muscles. *Brain*, **41**, 332–343.

WHITESIDE, T. C. D. (1952). Accommodation of the human eye in a bright and empty visual field. *J. Physiol.* **118**, 65*P*.

J. Physiol. (1961), **156**, *pp.* 578–590
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THE EFFECT OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND ACETYLCHOLINE ON CUTANEOUS AFFERENT FIBRES

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(*Received* 5 *December* 1960)

The cutaneous nociceptors have thin myelinated and non-myelinated afferent fibres (see Zotterman, 1959 for a review), but there is little information about the sensitivity of the endings of individual fibres to cutaneous stimuli. This arises because the small diameters of their afferent fibres have delayed an exact analysis with single-fibre techniques and also because stimuli sufficient to excite the nociceptors will, in addition, not only excite the receptors highly sensitive to the particular stimulus but also excite receptors which are highly sensitive to other forms of energy, i.e. the specificity of the nerve endings is not absolute. Identification of the nociceptors is thus made more difficult. All the non-myelinated fibres, in particular, have come to be regarded as pain fibres, even though Zotterman (1939) had described a discharge of small impulses, which were presumed to be in non-myelinated cutaneous fibres, when only mild mechanical stimuli were used. More recently Douglas & Ritchie (1957*b*) recording from intact nerves, and Iggo (1960) using single-fibre techniques proved that many C fibres could be excited by mild mechanical stimulation of the skin. A quantitative examination of single units established that the mechanically sensitive C fibres had a wide range of thresholds, from 20 mg to more than 7·5 g. Other C fibres were specifically sensitive either to heating or to cooling of the skin (Iggo, 1959*b*; Hensel, Iggo & Witt, 1960; Iriuchijima & Zotterman, 1960). No units could accurately be identified as nociceptors, although the thresholds to all stimuli of the least sensitive were so high that the effective stimuli could be regarded as noxious. For this reason additional more specific tests are required in order to identify the nociceptors.

If all cutaneous nociceptors were excited by all noxious agents, as is generally assumed, then the injection into the skin of pain-producing chemicals might provide such an additional test. Histamine, acetylcholine, 5-hydroxytryptamine (5-HT) and mixtures of histamine and acetylcholine will in low concentrations (10^{-3}–10^{-8} g/ml.), cause pain when put on blister bases in man (Armstrong, Dry, Keele & Markham, 1953), as will histamine when it is injected into the superficial layer of the skin

(Rosenthal, 1950) and a mixture of histamine and acetylcholine when it is pricked into the skin (Emmelin & Feldberg, 1947; Skouby 1953). Histamine when put on frog skin may provoke a discharge of impulses in afferent fibres (Habgood, 1950; Dodt, 1955; Arthur & Shelley, 1959) as will acetylcholine in the appropriate conditions (Jarrett, 1956). In mammals, on the other hand, Brown & Gray (1948), Douglas & Gray (1953), Witzleb (1959) and Douglas & Ritchie (1960) excited some of the fibres in multi-fibre strands of cutaneous nerves with the close-arterial injection of acetylcholine, but not with histamine.

The present paper describes an attempt to identify cutaneous nociceptors by making close-arterial injections of histamine, acetylcholine, histamine and acetylcholine and 5-hydroxytryptamine into the skin while recording from single-unit preparations. Preliminary accounts of the results have been published (Fjällbrant & Iggo, 1959; Iggo, 1959*a*).

METHODS

The eleven cats used were anaesthetized with chloralose, 80 mg/kg I.V., after induction of anaesthesia with ethyl chloride and ether. A skin incision was made in the mid thigh above the saphenous nerve. The femoral artery was then dissected in the thigh and the muscular branches to a point about 2 cm distal to the origin of the saphenous artery were ligated. A fine polythene cannula was inserted in a retrograde direction into the femoral artery in Hunter's canal so that its tip was just distal to the orifice of the saphenous artery. The femoral artery was occluded centrally during the injection, which was washed in with Ringer–Locke solution. The thoroughness of the ligation of the muscular branches was checked in some experiments by injecting Indian ink through the cannula at the end of the experiment.

The fixation of the leg with metal pins through each end of the tibia, the dissection of the single units, the methods used to provide quantitative mechanical and thermal stimulation of the skin and the recording methods were similar to those described previously (Iggo, 1959*b*, 1960).

Chemicals. The chemicals were stored dry and were dissolved in Ringer–Locke solution as required during each experiment: histamine acid phosphate (Burroughs Wellcome), acetylcholine chloride (Roche Bros. Ltd), 5-hydroxytryptamine creatinine sulphate (Abbott Laboratories) and impure bradykinin (a gift from Dr E. W. Horton). The amounts injected are expressed as the base, with the exception of bradykinin.

Method of injection. The close-arterial injections were made into the femoral artery at the orifice of the saphenous artery. The latter supplies blood to a large area of the skin on the medial aspect of the thigh, leg and foot. This procedure was adopted because the single fibres were dissected from the nerve in the mid thigh and the position of the receptive fields could not, therefore, be predicted. As a result, however, the conditions of the experiment differ from the similar investigations of Brown & Gray (1948), Douglas & Gray (1953) and Douglas & Ritchie (1960) in that the chemicals were put into the blood stream at a greater distance from the receptors in the present work and the blood flow was not restricted to the skin. The chemicals thus probably had, in the present work, a more gradually rising concentration at the nerve endings and some of the acetylcholine might also have been destroyed in the blood.

RESULTS

Multi-fibre strands of the saphenous nerve

Multi-fibre strands were used in the initial experiments because the actions of the injected chemicals could be tested on a large sample of afferent fibres. The identification of a particular active fibre was, however, usually unreliable when there were numerous fibres in a strand.

Histamine. The quantities used ranged from 6·6 to 230 μg. The largest doses were little more effective than 60 μg; in most experiments the doses ranged from 10 to 20 μg. These amounts are less than the quantities of histamine released rapidly in the skin by drugs such as compound 48/80 (Feldberg & Paton, 1951), and may therefore be regarded as within physiological limits.

The first reaction to an injection of histamine appeared within 5 sec with the largest doses and the peak of activity occurred at 1–3 min. The maximum frequency of discharge was low, never greater than 10/sec, and the impulses were often grouped in bursts. The activity did not persist beyond 6 min. Only a small proportion of the fibres in a multi-fibre strand were excited by the histamine.

Acetylcholine (Ach) was tested, in doses ranging from 20 to 60 μg, on six strands. Any discharge of impulses appeared soon after the injection, as reported by Brown & Gray (1948), Douglas & Gray (1953) and Douglas & Ritchie (1960). With one strand, containing both myelinated and non-myelinated fibres, there was a burst of activity, in a few of the fibres, which began before the injection was completed, reached a peak 20 sec after the start of the injection, fell quickly to low level at 35 sec and then persisted until 110 sec. On another occasion impulses which appeared to be in a single A fibre were recorded; the frequency was not greater than 16 sec at the peak of excitation and bursts of impulses were present for 4 min after the injection.

Histamine and acetylcholine. Mixtures of these chemicals when pricked into human skin were reported by Emmelin & Feldberg (1947) and Skouby (1953) to cause stinging pain. For this reason the mixtures were injected in the present work.

The combined injections were more potent than the injection of either chemical alone. The discharge was maximal 1–3 min after the injection (Fig. 1) and bursts of impulses continued for as long as 10 min. The maximal frequency of discharge was not very high, and when it could be recognized as arising in particular fibres it was never more than 5/sec.

5-hydroxytryptamine. Douglas & Ritchie (1957*a*) found that 5 hydroxytryptamine (5-HT) was very potent in exciting the cutaneous afferent

fibres. This is confirmed by the present work. Injection of 4–40 μg of 5-HT aroused a discharge which began within 20 sec, reached its peak at 1–3 min and persisted on one occasion for as long as 13 min. The discharge was, as with the other chemicals, intermittent; groups of impulses were separated by longer intervals of silence.

The characteristic response to injection of all the above chemicals was,

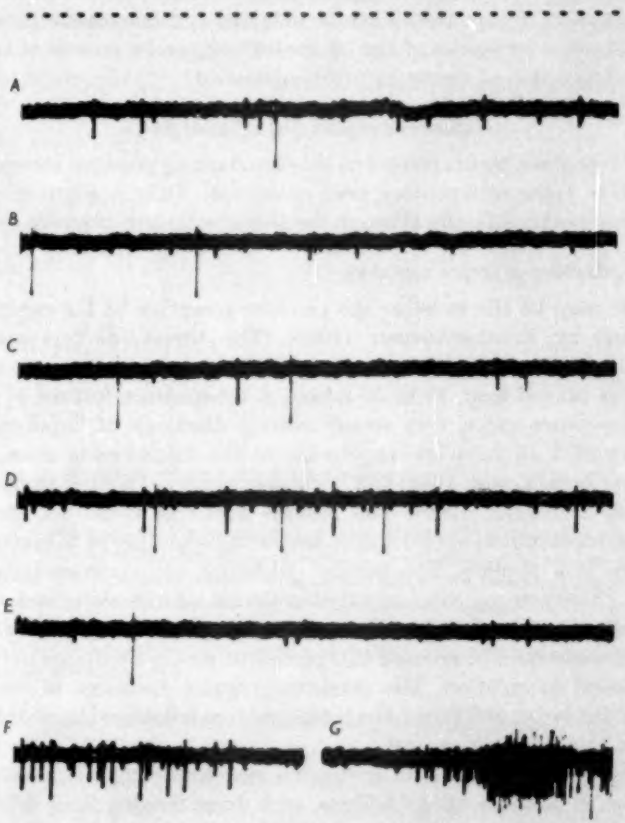

Fig. 1. The effect of an injection of a mixture of histamine (20 μg) and acetylcholine (40 μg) on a multi-fibre strand of the saphenous nerve. The base line before the injection, not shown, was silent. The records from above down were taken at 15 sec, 30 sec, 45 sec, 1 min 45 sec and 3 min 45 sec after the injection. The rate of firing in individual fibres did not exceed 5/sec and only a small proportion of all the fibres in the strand were excited. An indication of the number of fibres in the strand can be gauged from the response to (*F*) heating the skin and (*G*) stroking the skin firmly. Time marker, 0·1 sec, applies to all tracings.

therefore, a low-frequency sporadic discharge of impulses in only a few of the fibres in multi-fibre strands of the saphenous nerve.

Action on single afferent fibres

An important advantage gained by the use of single units, combined with quantitative natural stimulation of the receptors, is that not only can any excitatory action of the chemicals be measured accurately but also any depression of excitability can be detected and measured. In addition the conduction velocities of the afferent fibres can be measured and thus give an indication of the types of fibres studied.

Cutaneous myelinated afferent fibres

Hair receptors, touch receptors, slowly-adapting pressure receptors and insensitive pressure receptors were examined. Only one group of these receptors was found to be affected, the slowly-adapting pressure receptors.

Slowly-adapting pressure receptors

These may be the same as the pressure receptors in the rabbit's skin described by Frankenhaeuser (1949). The thresholds for mechanical stimulation of the receptors were about 1 g wt. and the conduction velocities ranged from 20 to 55 m/sec. A conspicuous feature of some of these receptors was a very steady resting discharge of impulses at frequencies of 3–15 impulses/sec, similar to the discharge in some corneal afferent fibres (Tower, 1940) and in cutaneous fibres (Witt & Hensel, 1959; Hunt & McIntyre, 1960). The resting discharge could be altered by mechanical stimulation of the skin, increasing smoothly in frequency when pressure was applied. The resting discharge was depressed for a few seconds after strong mechanical stimulation of the skin, and returned gradually during a few seconds to the resting level. If a unit was silent it could sometimes be aroused to a persistent steady discharge by vigorous mechanical stimulation. The persistent regular discharge of these units was particularly useful since the receptors were influenced by the chemicals used in this work.

Histamine. The action of histamine was consistent. It was recorded 19 times in 21 trials using 11 fibres, with doses ranging from 6·6 to 66 μg. In the later experiments in the series 10 μg was invariably effective. The effect on the resting discharge of one fibre is illustrated in Fig. 2 (open circles). Within 20 sec of the injection the resting discharge began to increase and was maximal at 1–3 min. The discharge then stopped, more rapidly than it had increased. It was absent for 2 min. The silence in other fibres lasted from 10 sec to 12 min. Recovery to the original value was gradual, taking as long as 30 min. In one experiment the resting

discharge was permanently enhanced after repeated injections. If a fibre was silent before the injection a discharge of impulses could be evoked, with a time course similar to that for the active fibres.

The response of the same unit to an intermittently applied mechanical stimulus passed through a similar cycle of enhancement, depression and recovery when histamine was injected. The initial enhancement was most obvious at the time when the resting discharge was failing. The recovery from the depression was also earlier for the response to mechanical stimulus than for the resting discharge, and there was a period of enhancement during the recovery (Fig. 2, filled circles). Two interesting features of the

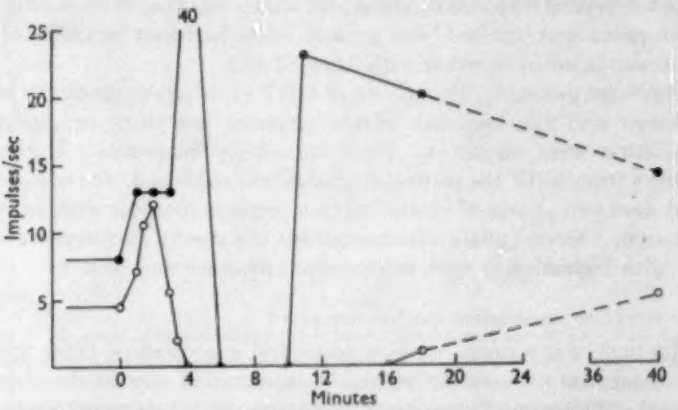

Fig. 2. The effect on an A pressure receptor (threshold, 1·25 g wt. conduction velocity, 35 m/sec) of an injection of 66 μg histamine. ○—○ resting discharge, ●—● the response to intermittent pressure with a small probe. The stimulus intensity chosen was less than twice threshold intensity. The maximal response to mechanical stimulation coincided with the failure of the spontaneous discharge, but, as described in the text, the discharge during mechanical stimulation was not as persistent during this phase as it was normally.

enhancement were, (1) the units did not continue to discharge throughout the period of mechanical stimulation as they did normally, there was an apparent acceleration of adaptation, and (2) the firing became intermittent, with gaps that initially were a multiple of the pulse interval. These gaps are unlikely to have been due to a failure of the impulses to leave the nerve terminals, since, initially, the normal interval was just doubled. This response implies that the usual sequence of events at the nerve ending leading to and following the discharge of an impulse had taken place, but that the impulse did not travel to the recording electrodes.

The response to a second injection of histamine during the depression associated with an initial dose was not examined.

Acetylcholine. Only two units were tested. One was unaffected by doses of 30 and 60 μg; the other was excited by 30 μg. Within 30 sec of the start of injection the frequency of the resting discharge was enhanced and it reached a maximum at 3–4 min (Fig. 8 in Iggo, 1959*a*). Twelve minutes later the discharge had fallen to its original value. This is a very much more prolonged action than was reported for ACh by Brown & Gray(1948) and Douglas & Gray (1960). The persistence of the effect suggests that it might have been due to a secondary action of the ACh.

Histamine and acetylcholine. The combination of ACh with histamine tended to intensify the action of histamine (Fig. 8 in Iggo, 1959*a*). In this figure the typical response to histamine, which was absent when only 20 μg of histamine was injected, was present when half that quantity of histamine was injected together with 24 μg of ACh.

5-hydroxytryptamine. The effects of 5-HT (1–20 μg) on both the resting discharge and the response of the pressure receptors to mechanical stimulation were similar to those caused by histamine. During the recovery from 5-HT the resting discharge was enhanced. On one occasion there were two phases of excitation of a pressure receptor with no resting discharge. A second phase of enhancement of a steady discharge was never seen with histamine or with mixtures of histamine and ACh.

Hair receptors, insensitive mechanoreceptors

Although many nerve strands contained examples of these types of unit there was no occasion when the injection of any of the chemicals aroused a discharge of impulses in the fibres. Five hair receptors and two insensitive mechanoreceptors with myelinated afferent fibres, isolated as single units, were tested with histamine and with histamine and ACh. No change in the response to quantitative mechanical stimulation was found.

Cutaneous non-myelinated afferent fibres

Mechanoreceptors

Ten single mechanoreceptors with non-myelinated afferent fibres were examined and included examples of all the Groups I, II and III (Iggo, 1960).

Histamine. Only two of the ten units were excited by the injection of histamine (10–160 μg). The largest response was a few impulses, at less than 5/sec, within 30 sec of the injection. An immediate short-lived enhancement of the response to mechanical stimulation may have been missed because the units were not tested at frequent intervals. In order to obtain a steady base line the mechanical stimuli could not be repeated more frequently than once every 30 sec, because more frequent stimulation leads to an inexcitability of the nerve endings (Iggo, 1960). The two units

tested most thoroughly both revealed a persistent depression after histamine (Fig. 3); one unit did not recover its original excitability for 1 hr. The other four units were apparently unaffected by histamine in the quantities injected.

Acetylcholine. Only one of the four units examined was excited by 30 μg of ACh; other unidentified units in the same strand fired intermittently, at frequencies not greater than 6/sec, for 5–30 sec after the injection. With one of the units there was an apparent twofold enhancement of the response to mechanical stimulation 30 sec after the injection.

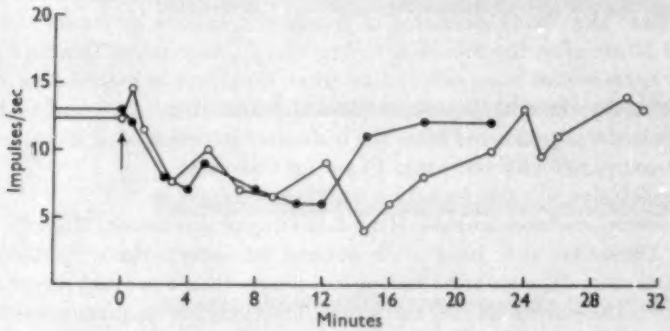

Fig. 3. The effect of histamine 10 μg ○—○ and of a mixture of histamine, (10 μg) and acetylcholine (24 μg) ●—● on the response of a C mechanoreceptor to pressure on the skin with a nylon probe, tip diameter 0·25 mm. The threshold of the unit was 1·5 g wt. At no time was there a discharge of impulses in the absence of a stimulus.

Histamine and acetylcholine. The mixture, which was tested on seven mechanoreceptors with non-myelinated afferent fibres, was more effective than the injection of either chemical alone. A low frequency discharge at 4–5 imp/sec was the largest response, but brief bursts of impulses did persist for as long as 14 min. The response to monitored mechanical stimulation was enhanced briefly by as much as 50% with three of the seven units. A later depression was evident with six of the seven units and lasted for as long as 30 min (Fig. 3).

5-hydroxytryptamine. One of the two units tested was excited by 2 μg of 5-HT. The discharge was weak, about 1/sec. The response to mechanical stimulation was simultaneously enhanced and during the depression which followed the enhancement it fell to 25% of the control value.

Thermoreceptors

Only the less sensitive thermoreceptors with non-myelinated fibres (Iggo, 1959*b*) were tested.

Histamine. The one *heat receptor* tested was excited by histamine, and 6 min after the injection of 8·3 μg of histamine there were still bursts of impulses. The rate of firing was, however, low, less than 5/sec. The mechanical sensitivity of this unit showed a brief but pronounced enhancement 15 min after the injection of 66 μg of histamine. A brief stroking of the skin, which initially was insufficient to excite the unit, evoked a discharge of impulses at 14/sec which persisted at a slowly declining frequency for at least 10 sec. This response was most unusual. Later testing with the same mechanical stimulus again failed to excite the unit.

The one *cold unit* tested was depressed by the injection of 100 μg of histamine; the steady discharge of impulses produced by ice on the skin ceased 20 sec after the injection and returned 73 sec later. This unit gave a very conspicuous burst of impulses when the skin was heated after being kept at a low temperature (the 'rewarming burst', Iggo, 1959*b*). This burst of impulses was prolonged after the histamine injection, and lasted nearly 60 sec compared with the usual 15 sec for this unit.

Acetylcholine was not tested on the thermoreceptors.

Histamine and acetylcholine. One *heat receptor* was tested. Injections of 10 μg histamine and 20 μg ACh evoked an intermittent discharge of impulses (Fig. 4), each burst lasting not longer than 2 sec and recurring at variable intervals for as long as 14 min. The maximal frequency was 5/sec. The response to thermal stimulation was depressed after the injection (Fig. 4) even when there were intermittent bursts of impulses, with recovery after about 10 min.

5-hydroxytryptamine. The *heat receptor* tested was excited by 2 μg of 5-HT. Bursts of impulses at low frequency were present for 4 min. The response to thermal stimuli, tested at 3 min intervals, was depressed in much the same way as by histamine and ACh.

Intradermal injections of histamine and of 5-hydroxytryptamine. Histamine was pricked or rubbed into the skin in the receptive fields of two slowly-adapting pressure receptors with myelinated afferent fibres. On each occasion there was an enhancement of the resting discharge, twofold with one unit and fourfold with the other. The peak frequency fell gradually to the resting level after 38 and 8 min, respectively. 5-HT was pricked into the skin in the receptive fields of two mechanoreceptors with non-myelinated afferent fibres and each time the units became unresponsive to mechanical stimulation.

Other observations

Bradykinin (impure) was injected in doses of 300–400 μg in three cats. The eight slowly-adapting pressure receptors with myelinated afferent fibres which were tested were all excited by the injection. A steady resting

discharge of 7·5/sec in one fibre rose after 25 sec to 20/sec, remained at this level for 6 min, and slowly fell to the original value after 30 min. Non-myelinated fibres in the same strand were excited only very feebly if at all.

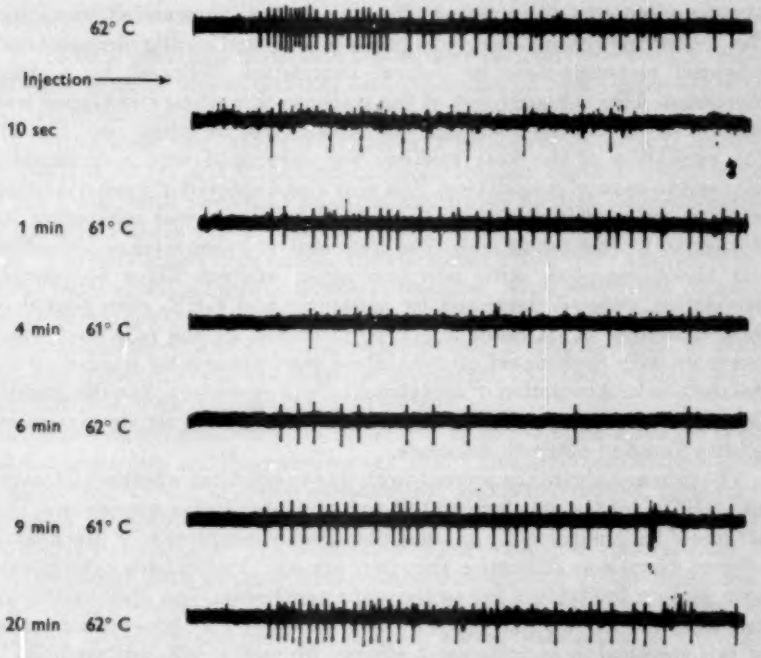

Fig. 4. The effect of an injection of histamine, (10 μg) and acetylcholine, (20 μg) on a C heat receptor. Before the injection there was a brisk response when a brass rod, 10 mm diameter, at 62° C was placed on the skin. After the injection this response was progressively diminished and was minimal at 4–6 min. Twenty minutes later the thermal response was almost normal. Ten seconds after the injection there was a burst of impulses in the fibre in the absence of heat (the record was taken with twice the gain of the other records in order to reveal the activity aroused in other fibres by the injection). The irregular discharge at very low frequencies recurred for 6 min and was present even when the sensitivity to the thermal stimulus was low. The temperature of the rod, which was placed on the skin near the start of each record except the second and left on for the rest of the trace, and the times of application, are indicated at the left. The injection was made at zero time. Spikes retouched. Time marker, 0·1 sec.

DISCUSSION

The various cutaneous afferent fibres were not uniformly sensitive to the intra-arterial histamine, histamine and acetylcholine, and 5-HT. There was a definite excitatory action on the slowly-adapting pressure receptors with myelinated afferent fibres and on the two heat receptors with non-myelinated afferent fibres tested. There were other unidentified responses. The pressure receptors displayed a phase of increased resting discharge and enhanced responsiveness to natural stimulation, followed by a deep depression. This enhancement of the response to natural stimulation was greatest as the resting discharge was falling after its initial rise (Fig. 2). The excitation of the heat receptor was associated with a diminished response to natural stimulation. This unit also displayed a much enhanced response to mechanical stimulation at the time it was recovering its sensitivity to thermal stimuli. The responses of other mechanoreceptors and thermoreceptors with non-myelinated afferent fibres to natural stimulation were all depressed by histamine and 5-HT, even though a weak discharge of impulses might be provoked by the injection. Hair receptors with myelinated afferent fibres were apparently unaffected by the chemicals. Any action of acetylcholine was excitatory, but the quantities injected were apparently inadequate to reveal the strong excitatory actions found in other experiments.

The primary aim in the present work was to establish whether histamine and 5-HT would be satisfactory test agents in identifying nociceptors. The actions of both chemicals in exciting the pressure receptors with myelinated afferent fibres is an indication that they are not. Preliminary experiments with impure bradykinin led to the same conclusion. The unsuitability of acetylcholine for this purpose is evident from the very generalized action of this chemical in exciting most afferent fibres if a sufficient quantity is used (see Paintal, 1956, for a review). Douglas & Ritchie (1960) have recently described an excitant action of acetylcholine on cutaneous non-myelinated fibres. The present experiments in which there was no conspicuous discharge of impulses following acetylcholine, but in which histamine and acetylcholine were effective, are more comparable with the sensory studies of Emmelin & Feldberg (1947) and Skouby (1953), since these investigators made intracutaneous injections of acetylcholine which by itself had no sensory effect, yet caused a change in the response to histamine when both chemicals were injected together.

Until more is known of the properties of the least sensitive cutaneous receptors it is premature to claim that the nociceptors have been satisfactorily identified. Certainly no fibres with a more or less uniform sensitivity to all noxious stimuli, including the pain-producing chemicals, have

yet been found. Instead, there are indications that the nociceptors may be modality-specific, since insensitive cold receptors, heat receptors and mechanoreceptors with non-myelinated afferent fibres have been described (Iggo, 1959*b*, 1960). The idea that the chemicals we have used could pick out all the nociceptors may thus be ill-founded.

SUMMARY

1. Close-arterial injections into the saphenous artery were made while electrical activity was recorded in cutaneous afferent fibres dissected from the saphenous nerves in cats anaesthetized with chloralose.

2. Histamine (6·6–230 μg), 5-hydroxytryptamine (4–40 μg), acetylcholine (20–60 μg) and histamine and acetylcholine excited briefly some of the fibres, both large and small, in multi-fibre strands. Identification of the active fibres was not usually possible.

3. The only single myelinated afferent fibres which were affected were those from slowly-adapting mechanoreceptors. After injections of histamine (6·6–66 μg), 5-hydroxytryptamine (1–20 μg) and of histamine and acetylcholine there was an initial enhancement, lasting 2–4 min, of both the resting discharge and the response to pressure, followed by a depression of sensitivity, with recovery after 10–60 min.

4. The non-myelinated fibres examined as single units included both mechanoreceptors and thermoreceptors. Some heat and mechanoreceptors were excited by the injection of histamine and acetylcholine, histamine and 5-hydroxytryptamine, but the rate of firing was never greater than 5/sec and was sporadic. The sensitivity of the endings to natural stimuli was depressed, even in those fibres which were excited by the injections.

5. Impure bradykinin enhanced the steady resting discharge of slowly-adapting A pressure receptors but had only a very weak action on C fibres.

REFERENCES

ARMSTRONG, D. R. M. L., KEELE, C. A. & MARKHAM, J. W. (1953). Observations on chemical excitants of cutaneous pain in man. *J. Physiol.* **120**, 326–351.

ARTHUR, R. P. & SHELLEY, W. B. (1959). The peripheral mechanism of itch in man; in *Ciba Foundation Study Group No.* 1. *Pain and Itch. Nervous Mechanisms*, pp. 84–95. London: Churchill.

BROWN, G. L. & GRAY, J. A. B. (1948). Some effects of nicotine-like substances and their relation to sensory nerve-endings. *J. Physiol.* **107**, 306–317.

DODT, E. (1955). Aktivierung markhaltiger und markloser Schmerzfasern bei Temperaturreizen. *Pflüg. Arch. ges. Physiol.* **260**, 225–238.

DOUGLAS, W. W. & GRAY, J. A. B. (1953). The excitant action of acetylcholine and other substances on cutaneous sensory pathways and its prevention by hexamethonium and D-tubocurarine. *J. Physiol.* **119**, 118–128.

DOUGLAS, W. W. & RITCHIE, J. M. (1957*a*). Discharges on non-myelinated afferent fibres in the cat's saphenous nerve in response to touch and to drugs. *J. Physiol.* **139**, 9–10*P*.

DOUGLAS, W. W. & RITCHIE, J. M. (1957*b*). Non-medullated fibres in the saphenous nerve which signal touch. *J. Physiol.* **139**, 385–399.

DOUGLAS, W. W. & RITCHIE, J. M. (1960). The excitatory action of acetylcholine on cutaneous non-myelinated fibres. *J. Physiol.* **150**, 501–514.

EMMELIN, N. & FELDBERG, W. (1947). The mechanism of the sting of the common nettle (*Urtica urens*). *J. Physiol.* **106**, 440–455.

FELDBERG, W. & PATON, W. D. M. (1951). Release of histamine from skin and muscle in the cat by opium alkaloids and other histamine liberators. *J. Physiol.* **114**, 490–509.

FJÄLLBRANT, N. & IGGO, A. (1959). Cutaneous afferent fibres and pain-producing chemicals. *J. Physiol.* **145**, 28–29*P*.

FRANKENHAEUSER, B. (1949). Impulses from a cutaneous receptor with slow adaptation and low mechanical threshold. *Acta physiol. scand.* **18**, 68–74.

HABGOOD, J. S. (1950). Sensitization of sensory receptors in the frog's skin. *J. Physiol.* **111**, 195–213.

HENSEL, H., IGGO, A. & WITT, I. (1960). A quantitative study of sensitive cutaneous thermoreceptors with C afferent fibres. *J. Physiol.* **153**, 113–126.

HUNT, C. C. & MCINTYRE, A. K. (1960). Properties of cutaneous touch receptors in cat. *J. Physiol.* **153**, 88–98.

IGGO, A. (1959*a*). A single unit analysis of cutaneous receptors with C afferent fibres; in *Ciba Foundation Study Group No.* 1. *Pain and Itch. Nervous Mechanisms*, pp. 41–56. London: Churchill.

IGGO, A. (1959*b*). Cutaneous heat and cold receptors with slowly-conducting (C) afferent fibres. *Quart. J. exp. Physiol.* **44**, 362–370.

IGGO, A. (1960). Cutaneous mechanoreceptors with afferent C fibres. *J. Physiol.* **152**, 337–353.

IRIUCHIJIMA, J. & ZOTTERMAN, Y. (1960). The specificity of afferent cutaneous C fibres in mammals. *Acta physiol. scand.* **49**, 267–278.

JARRETT, A. S. (1956). The effect of acetylcholine on touch receptors in frog's skin. *J. Physiol.* **133**, 243–254.

PAINTAL, A. S. (1956). Excitation of sensory receptors in thoracic and abdominal viscera. *XX int. physiol. Congr. Rev.* 78–89.

ROSENTHAL, S. R. (1950). Histamine as possible chemical mediator for cutaneous pain. Dual pain response to histamine. *Proc. Soc. exp. Biol., N.Y.*, **74**, 167–170.

SKOUBY, A. P. (1953). The influence of acetylcholine, curarine and related substances on the threshold for chemical pain stimuli. *Acta physiol. scand.* **29**, 340–352.

TOWER, S. S. (1940). Units for sensory reception in the cornea; with notes on nerve impulses from sclera, iris and lens. *J. Neurophysiol.* **3**, 486–500.

WITT, I. & HENSEL, H. (1959). Afferente Impulse aus der Extremitätenhaut der Katze bei thermischer und mechanischer Reizung. *Pflüg. Arch. ges. Physiol.* **268**, 582–596.

WITZLEB, E. (1959). Zur Frage von cholinergischen Mechanismen bei der Erregung von afferenten Systemen. *Pflüg. Arch. ges. Physiol.* **269**, 439–470.

ZOTTERMAN, Y. (1939). Touch, pain and tickling: An electrophysiological investigation on cutaneous sensory nerves. *J. Physiol.* **95**, 1–28.

ZOTTERMAN, Y. (1959). The peripheral nervous mechanism of pain: a brief review; in *Ciba Foundation Study Group No.* 1. *Pain and Itch. Nervous mechanisms*, pp. 13–34. London: Churchill.

J. Physiol. (1961), **156**, *pp.* 591–602
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THE DISTRIBUTION, CONNEXIONS AND HISTOLOGY OF BARORECEPTORS IN THE PULMONARY ARTERY, WITH SOME OBSERVATIONS ON THE SENSORY INNERVATION OF THE DUCTUS ARTERIOSUS

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A previous paper (Coleridge & Kidd, 1960) presented electrophysiological evidence for the existence of baroreceptors in the pulmonary artery of the dog. The further experiments described in this paper provide more detailed information about the distribution of these receptors in the different parts of the pulmonary artery and about the intrathoracic pathways of their afferent nerves in reaching the cervical vagi. The sensory endings from which recorded impulses originated have also been identified histologically and described.

In the course of these experiments some observations on the adult dog seemed to suggest that baroreceptors might also be found in the ductus arteriosus. Therefore action potentials were recorded from afferent vagal fibres in piglets and puppies in a search for such receptors.

A brief account of some of these findings has already been published (Coleridge, Kidd & Sharp, 1961).

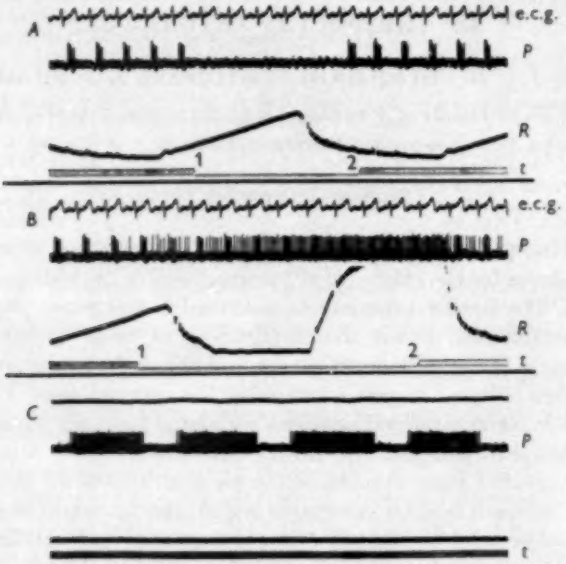
METHODS

Experiments were performed on dogs, puppies (4 weeks old) and piglets (3–8 days old). The dogs were anaesthetized with ether and chloralose (0·08 g/kg intravenously) or with morphine sulphate (3 mg/kg subcutaneously) followed by 0·25 ml./kg (intravenously) of a 1:1 mixture of Dial Compound (allobarbitone-urethane, Ciba) and sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd.) solutions. The puppies and piglets were anaesthetized with sodium pentobarbitone solution intraperitoneally (puppies 25–40 mg/kg); in piglets the dose producing anaesthesia was extremely variable (25–90 mg/kg).

The lungs were ventilated by a Starling 'Ideal' pump. The sternum was split in the mid line and the pericardium was opened. Respiration was recorded with an optical manometer attached to a side arm on the tracheal cannula. Action potentials were recorded from afferent fibres dissected from the right or left cervical vago-sympathetic trunk. An electrocardiogram was also recorded. A detailed account of the recording techniques has been given in a previous paper (Coleridge & Kidd, 1960).

Location of pulmonary arterial baroreceptors. The site of a receptor, from which action potentials were being recorded, was explored first by tightening and releasing, in turn,

ligatures placed round the origin of the main pulmonary artery and lung roots. The records shown in Text-fig. 1*A*, *B* depict the characteristic alterations in impulse activity by which pulmonary arterial baroreceptors were identified. The receptor was located accurately by exploring the artery with a fine probe after the animal had been killed and the vessels dissected; for several minutes after the cessation of the circulation direct mechanical stimulation of the receptor continued to produce action potentials in the vagal slip. Finally,



Text-fig. 1. Steps in the location of a pulmonary arterial baroreceptor for subsequent histological identification. *A*, effect of occlusion (1) and release (2) of the main pulmonary trunk. *B*, effect of occlusion (1) and release (2) of both lung roots (note increase in tracheal pressure during occlusion). The animal was killed and by direct pressure with a probe the receptor was located in the right branch just beyond the main bifurcation. A flap of the vessel wall containing the receptor was then cut (as described in the text). *C*, effect of punctate stimulation of the flap after it had been reduced to a 2 mm square. (Pl. 1, figs. 3, 4, shows the histological appearance of two endings found within this specimen.) In this and subsequent figures the following abbreviations are used: e.c.g., electrocardiogram; *P*, action potentials from vagal slip; *t*, time trace (1/50 sec); *R*, tracheal pressure (upstroke representing inflation); *S*, signal marker.

to make more certain the histological identification of the receptor, a small flap of the arterial wall was isolated by cutting round three sides of a square containing the receptor, leaving intact the direction in which the afferent fibre was expected to pass. Then, if action potentials could still be recorded, the flap was gently retracted and gradually reduced in size with fine scissors while the position of the receptor was checked repeatedly by probing (Text-fig. 1*C*). The discharge was finally abolished when the fourth side of the square was cut. In two experiments a receptor was thus localized to within a 2 mm square.

Nerve connexions of the pulmonary baroreceptors. In early experiments, seeking to deter-

mine the intrathoracic branches by which afferent fibres from pulmonary baroreceptors reached the cervical vagus, potentials were recorded from the branches themselves. The vagal trunk was placed on an earthed stainless-steel plate to reduce interference from the e.c.g. and impulses showing the behaviour characteristic of pulmonary arterial receptors were recorded from several such preparations. However, the fibres were often pulled from the electrodes by the movements of the heart or by the manoeuvres employed to locate the receptors. The movements also caused large artifacts in the electroneurogram.

The following alternative method was more satisfactory. The vagal branches in the chest were freed and fine threads were passed round them. Action potentials were recorded from a pulmonary arterial fibre isolated from the cervical vagus in the usual way. The thoracic path of the fibre was then determined by placing each of the vagal branches in turn upon the platform (5 mm wide) of a cooling device (Bjurstedt, 1946) the temperature of which could be lowered and recorded until the branch was found, cooling of which caused disappearance of impulses from the cervical vagal slip.

Histological methods. Specimens of the pulmonary artery were fixed in 10% formol-saline, embedded in paraffin and sectioned serially at a thickness of 15 μ. The sections were then mounted on slides and stained by the silver method of Holmes (1947).

The distribution of cholinesterase was examined in specimens taken from the pulmonary artery of one dog. The material was fixed in cold 10% formol-saline for 6 hr, and frozen sections cut tangentially to the vessel wall at 40 μ were subjected to a modified Koelle technique for the localization of cholinesterase (Coupland & Holmes, 1957) at values of pH between 4·4 and 5·2. Both acetyl- and butyrylthiocholine substrates were used.

Another specimen of the artery was fixed in 2% osmium tetroxide for the detection of myelinated nerve fibres.

RESULTS

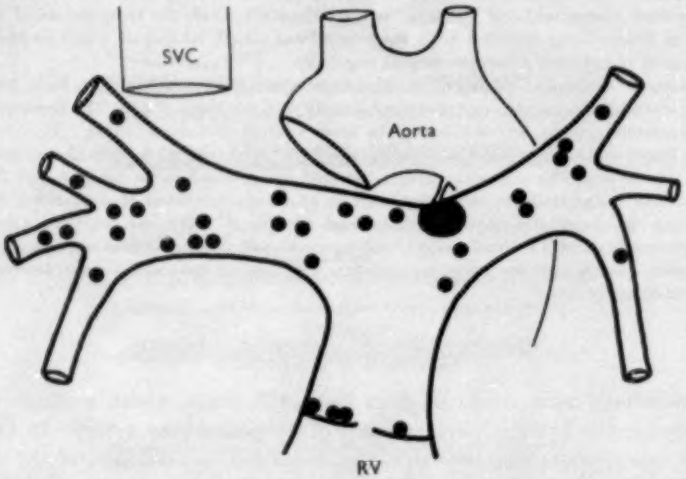
Location of receptors

Recordings were made in dogs from 128 fibres whose endings were situated in the extrapulmonary parts of the pulmonary artery. In 124 of these experiments the discharge was abolished by occlusion of the main pulmonary artery (Text-fig. 1*A*) and increased by obstruction of the lung roots (Text-fig. 1*B*); in 40 of these the exact position of the receptor in the pulmonary artery was determined precisely after death of the animal (Text-fig. 1*C*). As is depicted in Text-fig. 2, the majority of receptors lay in the region of the main bifurcation of the pulmonary trunk or in the right or left branches.

In a further four instances the discharge was increased by occlusion of the main pulmonary artery, and in each case after the animal had been killed the receptor was located proximal to the ligature in the first part of the pulmonary trunk just beyond the attachment of the valves. Otherwise the main trunk appeared to be devoid of receptors. To lessen any possibility that this was due to fibres being damaged by the occluding ligature, in thirty experiments the artery was occluded with the fingers.

In regard to the innervation of the different parts of the pulmonary artery by the two vagal nerves, the left vagus supplied endings in both the right and left arterial branches but there was no evidence that right vagal fibres crossed over to supply endings in the left pulmonary artery. Of the

20 receptors finally located precisely in the left pulmonary artery and round the attachment of the ligamentum arteriosum all the afferent fibres were in the left vagus. Of 20 endings found in the right pulmonary artery the afferent fibres were in the right vagus in 7 and in the left vagus in 13. (A further 8 receptors, innervated by afferent fibres in the right vagus, were stimulated by insertion of a probe into the right pulmonary artery after death of the animal, but impulse activity disappeared when the vessel was opened and their precise location was not determined.)



Text-fig. 2. The position of 44 pulmonary arterial baroreceptors from which action potentials had been recorded and whose location had been determined accurately by punctate stimulation after the animals had been killed and the pulmonary artery dissected. The position of 33 receptors is indicated by closed circles; the remaining 11 receptors were situated in the solid black area near the attachment of the ligamentum arteriosum. The ascending aorta and the superior vena cava (SVC) near its junction with the right atrium which lie anterior to the right pulmonary artery have been omitted for clarity.

Histological findings

The findings in the silver-impregnated sections will be described first. In two animals specimens were taken at random from the main pulmonary artery, the right branch and the left branch including the ligamentum arteriosum. In this randomly selected material no sensory endings of the type described below were found in either the main trunk or the left branch and ligamentum arteriosum. In the specimen of the right branch, however, no less than seven definite receptors were found. A typical example of one of these sensory endings is shown in Pl. 1, fig. 1. A single

myelinated nerve fibre in the adventitia approaches the tunica media obliquely, terminating at the junction of media and adventitia as an irregular collection of fine coiled fibres embedded in connective tissue. Plate 1, fig. 2, illustrates a second nerve ending in the same specimen, lying more deeply within the tunica media, close to the intima. Each of the endings detected in the randomly selected material was supplied by a thick myelinated axon, and lay either at the junction of media and adventitia, or within the media. No obvious receptors were seen within the adventitia away from the media.

Two specimens of the pulmonary artery were examined in which there was electrophysiological evidence for the presence of a baroreceptor. One was from the right branch; the action potentials recorded in this experiment are depicted in Text-fig. 1. This specimen contained four distinct and separate nerve endings of the type already described. Two of these are illustrated; one lies at the mid point of the media (Pl. 1, fig. 3), the other at the junction of media and adventitia (Pl. 1, fig. 4). Both are obviously supplied by myelinated fibres. The other specimen, in which a receptor had been located physiologically, was from the main pulmonary trunk just distal to the valves; only one sensory nerve ending was detected histologically in this specimen.

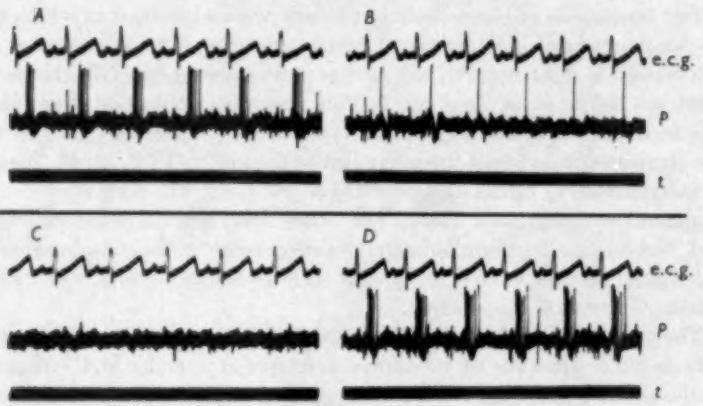
The specimens subjected to the histochemical method for cholinesterase revealed the presence of moderate amounts of specific and non-specific cholinesterase throughout the tunica media, in contrast to the connective tissue of the adventitia which was totally lacking in cholinesterase activity. Nerve fibres with a strongly positive cholinesterase activity were revealed in both media and adventitia, but only one possible example of a sensory ending was revealed by this method (Pl. 1, fig. 5). Here the diffuse precipitate of copper sulphide in the tunica media is visible, but there is a heavy concentration of precipitate over a small oval area within the media, indicating the presence of relatively large quantities of non-specific cholinesterase. The method does not permit accurate visualization of details of the structure, but it appears to be composed of a compact group of coiled fibres, and a fibre can also be seen approaching from the tunica adventitia. The over-all appearance is strongly reminiscent of the receptors revealed by the silver technique.

Many nerve fibres, other than those seen to terminate as specialized receptors, were observed in the silver-impregnated sections. Large bundles of fine non-myelinated fibres in the tunica adventitia could frequently be seen to follow the vasa vasorum passing into the tunica media, where fine non-myelinated fibres ran between the smooth-muscle fibres. Also present in the adventitia were occasional small groups of large ganglion cells lying within the bundles of non-myelinated fibres. Myelinated fibres

were, however, relatively few, as was confirmed by examination of the tissue fixed in osmium tetroxide.

Nerve connexions of the pulmonary baroreceptors

Text-figure 4 is a diagrammatic representation of the intrathoracic branches of the vagus nerves. It is based on our dissections in eight dogs and closely agrees with the description given by Mizeres (1955), whose terminology has been adopted in labelling Text-fig. 4.



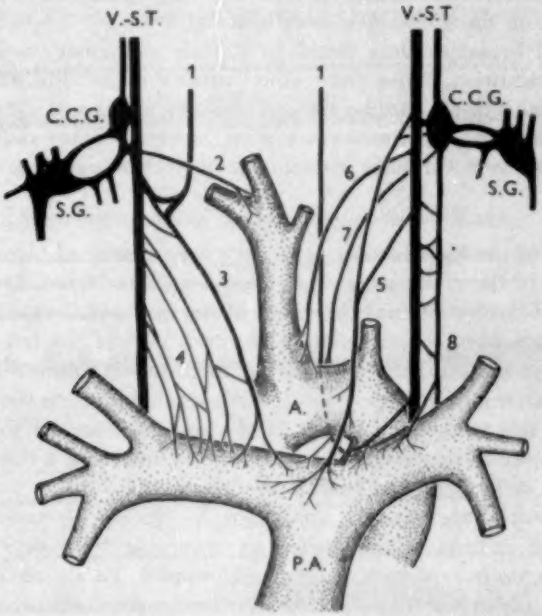
Text-fig. 3. Determination of connexions of pulmonary arterial baroreceptor. Action potentials recorded from slip dissected from right cervical vagus nerve. Recurrent cardiac nerve lifted from surrounding tissues and placed (intact) upon a cold-blocking device. The temperature of this device was then varied: *A*, 37° C; *B*, 9° C; *C* (a few seconds later), 9° C; *D*, 37° C.

In 10 out of 20 instances in which fibres from pulmonary arterial receptors were dissected from the left cervical vagus, impulses ceased when the ventromedial cervical cardiac nerve was blocked by cold, by the method described above and illustrated by the records depicted in Text-fig. 3. This nerve arises from the caudal cervical ganglion and adjacent vagal trunk. It receives additional filaments as it passes down between the brachiocephalic and left subclavian arteries to cross the ventral surface of the aortic arch and gain the pulmonary artery near the bifurcation.

Impulses in the remaining 10 left vagal fibres were abolished by cooling either the left recurrent laryngeal nerve, the ventrolateral cervical cardiac nerve or fine unnamed filaments which for part of their course run with these two nerves.

Ten pulmonary arterial fibres dissected from the right vagus were similarly investigated. Five were located in the recurrent cardiac nerve.

This is formed at about the level of the caudal cervical ganglion, receiving filaments from the ganglion, right recurrent laryngeal nerve and vagal trunk. It runs caudally and medially between the superior vena cava and



Text-fig. 4. Diagrammatic representation of the intrathoracic vagal branches which carry fibres from baroreceptors in the pulmonary artery. The terminology of Mizeres (1955) has been used. V.-S.T., vago-sympathetic trunk; C.C.G., caudal cervical ganglion; S.G., stellate ganglion; P.A., pulmonary artery; A, aorta. Branches of the vagal nerves: 1, recurrent laryngeal nerves; 2, right innominate nerve; 3, recurrent cardiac nerve; 4, vagal cardiac nerves; 5, ventromedial cervical cardiac nerve; 6, left innominate nerve; 7, dorsal cervical cardiac nerve; 8, ventrolateral cervical cardiac nerve. A branch which runs between the recurrent cardiac nerve and the left recurrent laryngeal nerve behind the aortic arch has been omitted for clarity. The ascending aorta, which lies anterior to the right branch of the pulmonary artery and the right vagal branches 3 and 4, has been omitted. Only a few of the many filaments of these branches which innervate structures other than the pulmonary artery have been depicted.

trachea, and sends branches to the right pulmonary artery. The remaining right vagal fibres passed behind the superior vena cava to the right pulmonary artery in the vagal cardiac nerves (usually 4 or 5 in number) which arise from the vagal trunk between the ansa subclavia and the level of the azygos vein.

Conduction in fibres from pulmonary arterial baroreceptors was blocked when the nerves were cooled to 8–10° C.

The presence of pulmonary arterial fibres in the ventromedial and ventrolateral cervical cardiac nerves was also demonstrated by direct recording from these branches (see Methods).

All vagal branches thus found to contain pulmonary arterial fibres carried, in addition, fibres from other sensory areas. For example, the ventromedial cervical cardiac nerve contained many aortic baroreceptor fibres; similarly, aortic baroreceptor, atrial, chemoreceptor and pulmonary stretch fibres were variously identified in the other branches.

Are there baroreceptors in the ductus arteriosus?

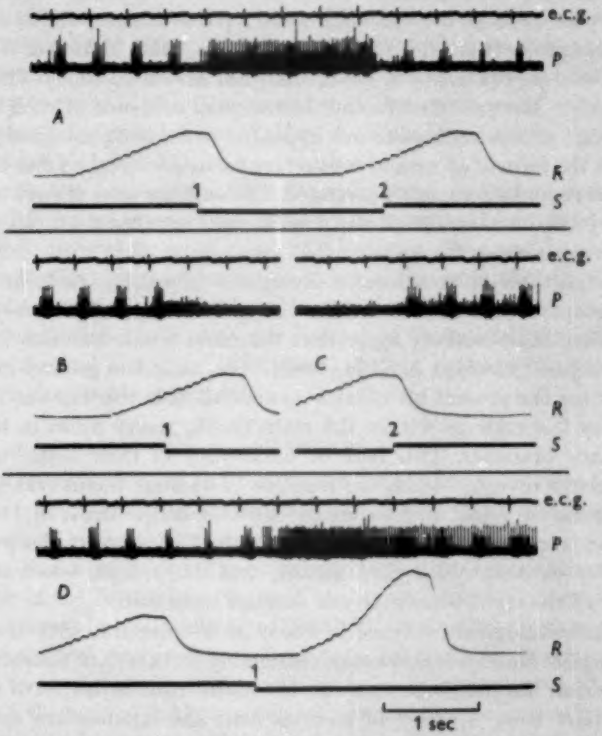
Endings of the baroreceptor type have been described histologically in the ductus of the new-born animal (Nonidez, 1935; Boyd, 1941). It was therefore of interest to find that 25% of the receptors located accurately in adult dogs were concentrated in the first part of the left pulmonary artery round the attachment of the ligamentum arteriosum (Text-fig. 2). In one instance a receptor was located in a small dimple on the endothelial surface of the pulmonary artery at the proximal end of the ligament. A high-frequency discharge was produced by inserting a fine probe into the dimple or by gently pulling on the ligament.

Consequently an attempt was made to obtain electrophysiological evidence of endings in the ductus by recording from slips of the left cervical vagus in new-born piglets and puppies. To aid identification of receptors, additional ligatures were placed round the ascending and descending aorta. It seemed likely that any receptors in the ductus would be subject not only to pressure changes in the ductus itself if the lumen were patent, but also to traction on the ductus due to pulsation of both the pulmonary artery and aorta. A search was therefore made for receptors with a cardiac rhythm from which a continuous discharge could be elicited by mechanical stimulation of the ductus.

Many receptors were found which could be stimulated by probing the ductus in the living animal. In some of these, however, occlusion in turn of different vessels produced such a complex sequence of alterations in impulse activity that location to one vessel in the living animal proved impossible, and detailed location after death was not attempted.

Even though a continuous discharge could be elicited by probing the ductus, all such receptors which were located accurately after the animal had been killed and the vessels dissected were found to be situated, not in the ductus itself, but in adjacent parts of the pulmonary artery and aorta. This is illustrated by the records shown in Text-fig. 5; there were at least three active fibres in this slip dissected from the left aortic nerve of a

puppy. In *A*, gentle pressure with a fine probe on the ductus produced a continuous discharge in all three fibres. Nevertheless, the alterations in impulse activity following occlusion of the pulmonary artery (*B*, *C*) and lung roots (*D*) suggested that the receptors were probably in the pulmonary artery. Finally, after the animal had been killed the endings of all



Text-fig. 5. Action potentials recorded from left aortic nerve in a puppy. There are three fibres showing a cardiovascular discharge in this slip. *A*, pressure with a fine probe on the ductus arteriosus (between 1 and 2). *B*, *C*, occlusion (1) and release (2) of origin of main pulmonary artery (interval of approximately 4 sec between *B* and *C*); the brief continuous discharge (smallest spikes) at the beginning of occlusion was probably caused by traction on the pulmonary artery. *D*, occlusion of lung roots (1); note increase in tracheal pressure during occlusion.

three fibres were located in the pulmonary arterial wall adjacent to the attachment of the ductus. Similarly, in other experiments, receptors which had been stimulated by gently probing the ductus were finally located in the concavity of the aortic arch to the right of the ductus.

In addition to the pulmonary arterial and aortic baroreceptor fibres already mentioned, impulses were also recorded from chemoreceptor, atrial and pulmonary stretch fibres in both puppies and piglets.

DISCUSSION

A number of workers have described branched sensory endings of the baroreceptor type in the extrapulmonary parts of the pulmonary artery of various species (Larsell & Dow, 1933; Takino, 1933; Takino & Watanabe, 1937; Nonidez, 1941; Boyd, 1941; Bianconi & Green, 1959). The present investigation has provided further histological evidence of such receptors in the dog; all the endings found appeared to be morphologically similar and took the form of an area of connective tissue occupied by fine branching coiled nerve fibres compactly arranged. The endings were always in contact with or buried within the tunica media; no receptors were recognized in the adventitia away from the media.

Although there is considerable divergence of opinion as to how widely these receptors are distributed in the pulmonary arteries beyond the bifurcation, most authors agree that the main trunk contains few if any receptors (see Coleridge & Kidd, 1960). Certainly the general impression gained from the present histological examination in the dog was that there were very few endings within the main trunk, many more in the extrapulmonary branches. This lack of uniformity in their distribution was more clearly revealed when the number of endings found was related to actual areas of vessel wall examined histologically. Thus, in 112 mm^2 of the main trunk only one ending (previously located near the pulmonary valves by electrophysiological means) was discovered, whereas in only 71 mm^2 of the right branch eleven endings were seen.

If this histological evidence is taken in conjunction with the electrophysiological findings it now seems reasonably certain that, apart from some endings near the pulmonary valves, the main trunk is devoid of receptors. Also, apart from a group of endings near the ligamentum arteriosum, the left branch appears to be more sparsely innervated than the right.

This distribution must be taken into account in the design of experiments to elucidate the functional significance of the pulmonary arterial baroreceptors. Also of interest in this connexion was the observation that half the pulmonary arterial baroreceptor fibres were concentrated, for part of their course, in just two branches of the vagus nerves, the ventromedial cervical cardiac nerve on the left and the recurrent cardiac nerve on the right. However, these branches also carried fibres from other sensory areas, which precluded electrical stimulation of these nerves as a means of determining function.

Our finding that both vagus nerves supply receptors in the right

pulmonary artery, whereas the left artery (including the arterial wall adjacent to the ligamentum arteriosum) is supplied only by the left vagus, is in agreement with previous evidence. Bianconi & Green (1959) recorded impulse activity in pulmonary baroreceptor fibres in the cat, and located the endings by mechanical stimulation. Their observations were confined to the right vagus, and while endings were found in the right artery and near the bifurcation none were located in the main trunk or left branch. On the other hand there is good anatomical evidence that the endings demonstrated histologically in the ductus arteriosus and adjacent left pulmonary artery of the new-born animal are innervated solely by the left vagus and aortic nerves (Takino & Watanabe, 1937; Boyd, 1941; Nonidez, 1941).

Although the left vagus and left aortic nerve in piglets and puppies contained fibres which were activated by traction on the ductus arteriosus, the endings proved in most cases to be within the aorta or pulmonary artery, and there were no instances in which we were able to obtain unequivocal electrophysiological evidence of endings within the ductus itself. Nevertheless, the histological findings of Takino & Watanabe (1937), Boyd (1941) and Nonidez (1935, 1941) clearly indicate the presence of endings in the ductus arteriosus.

SUMMARY

1. Electrophysiological and histological techniques have been employed in anaesthetized dogs to investigate the distribution and histological appearance of baroreceptors in the pulmonary artery.

2. Impulses were recorded from pulmonary baroreceptor fibres dissected from slips of the cervical vagus, and the exact position of the receptors was determined immediately after death by stimulation with a probe.

3. Histological examination of portions of tissue in which receptors had been located showed sensory endings in, or in contact with, the media. These took the form of fine branching coiled nerve fibres compactly arranged within an area of connective tissue, and were supplied by thick myelinated nerve fibres. Similar endings were found in specimens taken at random from the pulmonary artery.

4. The majority of endings were situated in the right pulmonary artery or in the left branch near the ligamentum arteriosum. Apart from a few endings near the pulmonary valves, the main pulmonary trunk was devoid of receptors.

5. The intrathoracic branches of the vagus which carry pulmonary baroreceptor fibres were determined by cooling the vagal branches near the heart. Half the fibres examined reached the right and left vagi via the recurrent cardiac and ventromedial cervical cardiac nerve respectively.

6. Observations in the adult dog suggested that baroreceptors might also be present in the ductus arteriosus, but attempts to confirm this by recording afferent impulses in new-born piglets and puppies were inconclusive.

We wish to thank Mrs Barbara James and Mr E. S. Stainthorpe for technical assistance, and Mr R. Adkin, A.I.S.T., for assistance in the preparation of the histological material.

REFERENCES

BIANCONI, R. & GREEN, J. H. (1959). Pulmonary baroreceptors in the cat. *Arch. ital. Biol.* **97**, 305–315.

BJURSTEDT, A. G. H. (1946). Interaction of centrogenic and chemoreflex control of breathing during oxygen deficiency at rest. *Acta physiol. scand.* **12**, Supplement 38.

BOYD, J. D. (1941). The nerve supply of the mammalian ductus arteriosus. *J. Anat., Lond.*, **75**, 457–468.

COLERIDGE, J. C. G. & KIDD, C. (1960). Electrophysiological evidence of baroreceptors in the pulmonary artery of the dog. *J. Physiol.* **150**, 319–331.

COLERIDGE, J. C. G., KIDD, C. & SHARP, J. A. (1961). Observations on the distribution, histology and nerve connexions of sensory endings in the pulmonary artery. *J. Physiol.* (in the Press).

COUPLAND, R. E. & HOLMES, R. L. (1957). The use of cholinesterase techniques for the demonstration of peripheral nervous structures. *Quart. J. micr. Sci.* **98**, 327–330.

HOLMES, W. (1947). The peripheral nerve biopsy. In *Recent Advances in Clinical Pathology*, ed. DYKE, S. C. London: Churchill.

LARSELL, O. & DOW, R. S. (1933). The innervation of the human lung. *Amer. J. Anat.* **52**, 125–146.

MIZERES, N. J. (1955). The anatomy of the autonomic nervous system in the dog. *Amer. J. Anat.* **96**, 285–318.

NONIDEZ, J. F. (1935). The aortic (depressor) nerve and its associated epithelioid body, the glomus aorticum. *Amer. J. Anat.* **57**, 259–301.

NONIDEZ, J. F. (1941). Studies on the innervation of the heart. II. Afferent nerve endings in the large arteries and veins. *Amer. J. Anat.* **68**, 151–189.

TAKINO, M. (1933). Vergleichende Studien über die histologische Struktur der Arteriae und Venae pulmonales, die Blutgefässnerven der Lunge und die Nerven der Bronchien bei verschiedenen Tierarten, besonders über die Beziehung der Blutgefässnerven zu den glatten Muskeln der Blutgefässe. III. Mitteilung. *Acta Sch. med. Univ. Kioto*, **15**, 321–354.

TAKINO, M. & WATANABE, S. (1937). Über die Bedeutung des Ligamentum arteriosum bzw. des Ductus Botalli und der Ansatzstelle desselben an der Pulmonalwand (A. pulm.) als Blutdruckzügler bei verschiedenen Tierarten. *Arch. Kreislaufforsch.* **11**, 18–27.

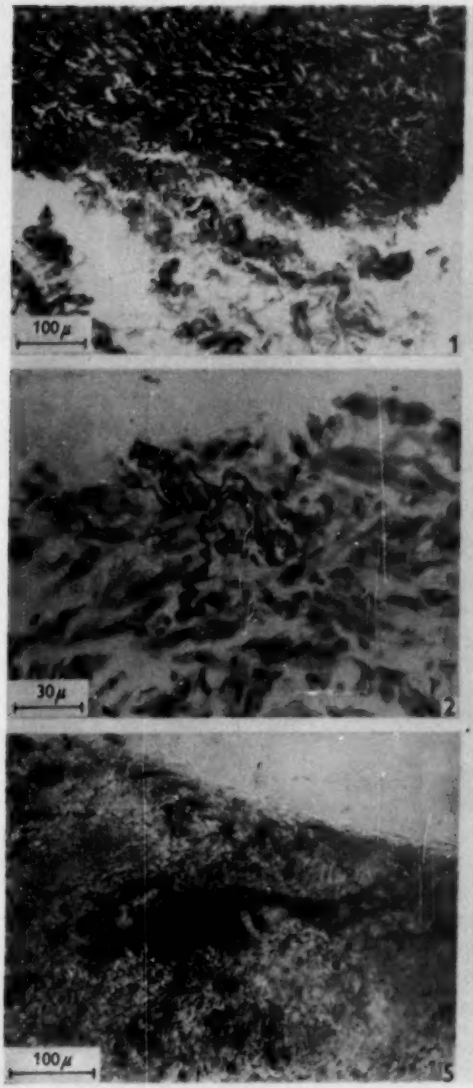
EXPLANATION OF PLATE

Fig. 1. Nerve ending in randomly selected material from right branch of pulmonary artery. A myelinated fibre approaches the tunica media (above) obliquely within the adventitia, terminating in a sensory ending at the junction of media and adventitia. Holmes's silver.

Fig. 2. Another nerve ending from the same specimen, lying within the inner part of the tunica media. Holmes's silver.

Figs. 3, 4. Two endings in a specimen of the right branch of the pulmonary artery. Electrophysiological evidence of a receptor in this specimen had previously been obtained (Text-fig. 1). Holmes's silver.

Fig. 5. Portion of tunica media showing diffuse positive reaction for non-specific cholinesterase. This contains a small area in which the reaction is strongly positive, revealing a structure similar to those displayed by the silver technique. Modified Koelle procedure for non-specific cholinesterase.



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TISSUE CHANGES ACCOMPANYING ACCLIMATIZATION TO LOW ATMOSPHERIC OXYGEN IN THE RAT

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Many of the circulatory and respiratory alterations which occur during acclimatization to low pressure of inspired oxygen are well established (Stickney & Van Liere, 1953). It has often been assumed that in the acclimatized state there is no alteration in the oxygen consumption of the whole body at rest and therefore no justification for anticipating changes in the oxygen consumption of isolated tissues. However, Hill (1959) has pointed out that many experimental reports would indicate that total oxygen consumption might be altered during the process of acclimatization.

There is no agreement between the few investigations into the oxygen consumption of isolated tissues taken from acclimatized rats. This may be due to the different techniques used to produce the acclimatized state or to the wide variety of tissues, substrates and oxygen levels used in the subsequent *in vitro* determinations, or to a combination of these and other factors.

Once the importance of the increase in circulating haemoglobin had been established by many early workers in this field, investigations followed into the role of other iron-containing respiratory pigments, i.e. myoglobin and the cytochromes. While there is general agreement concerning the changes in myoglobin levels, there still remains some question concerning the cytochrome c changes.

It was therefore of interest to measure both the oxygen consumption and cytochrome c concentration in tissues isolated from animals acclimatized for long periods to hypoxic conditions. A preliminary report of these experiments has been published (Duckworth, 1960).

METHODS

Wistar rats of approximately 50 g wt. were obtained from the Agricultural Research Station, Compton. They were divided into groups of the same sex. Each group consisted of equal numbers of control and test animals. The control animals were kept under normal laboratory conditions. The test animals were acclimatized to hypoxia by being kept in an atmosphere of lower O_2 content than atmospheric air.

The apparatus used is shown in Fig. 1. The oxygen content of the rat chamber was reduced by passing nitrogen through it until the required oxygen content was reached. Thereafter the oxygen level was kept constant by oxygen inflow through a water valve of the Müller type. This valve admitted oxygen as the total pressure inside the box fell, owing to absorption of the expired carbon dioxide by the 'Protosorb' (4–8 mesh; Siebe Gorman Ltd.) contained in a mesh cage in front of an electric fan. This fan ensured adequate circulation of the gas mixture inside the box. The oxygen was supplied to the Müller valve from a storage cylinder of high-purity oxygen, via a rotameter and a thin rubber storage bag; the bag was kept filled by adjustment of the oxygen inflow to a rate which was dependent upon the size and number of rats in the chamber.

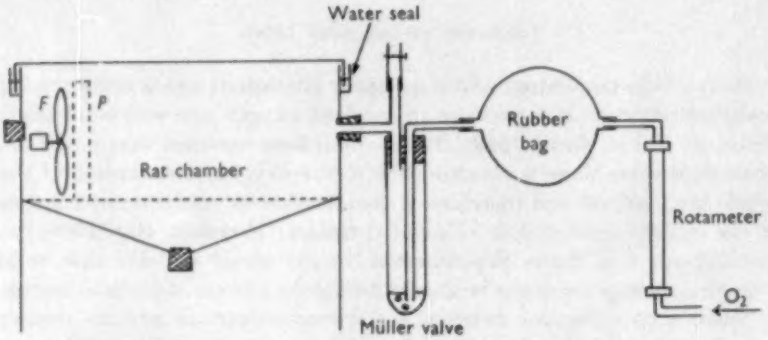

Fig. 1. Diagram of the apparatus for acclimatizing rats to low oxygen tension. *F* fan; *P* 'Protosorb'.

Groups of up to twenty rats were kept in this apparatus for an average of 6 months (range from 10 weeks to 10 months). At the start of each series the oxygen content was first reduced rapidly to 14 % (v/v) and then reduced slowly during the first week until the required level of 9–10 % oxygen was reached. The box was opened not more than once a day for cleaning and restocking with food (Diet 41, Bruce & Parkes, 1949), water and 'Frotosorb'.

Analysis of gases in the box was carried out twice daily in the Haldane apparatus. A record of the mean daily oxygen level for the first series is shown in Fig. 2. There is a fluctuation of the oxygen content due to impurities in the supplied oxygen and to variations in pressure within the box as a result of temperature changes.

Blood analysis. At the end of the period of acclimatization blood was obtained from the tail either by cutting the tip or directly from a tail vein into a heparinized syringe. Haemoglobin was estimated in the Haldane haemoglobinometer (100 % = 14·8 g Hb/100 ml.). The packed cell volume was measured by centrifuging at 3000 rev/min for 30 min in microhaematocrit tubes. These blood analyses were carried out both on the acclimatized rat and its control.

Tissue respiration. An acclimatized rat and its control were weighed; during subsequent procedures both animals were subjected to identical treatment in a paired experiment. Rats were killed by decapitation; the diaphragm was dissected out whole and bisected along the line of the central tendon. After trimming off the connective tissue, each hemidiaphragm was either used whole, or cut into two approximately equal pieces at right angles to its long axis. Excess fluid was removed by gently dabbing with filter paper and then each piece was accurately weighed on a torsion balance. The oxygen uptake of each piece was

measured simultaneously during incubation for 1½ hr at 38° C by the standard Warburg technique, in 1·6 ml. phosphate buffer (Kerly & Ottaway, 1954), to which 0·02 M lactate as sodium lactate was added. The gas phase was varied from 100 % oxygen to 2 % oxygen in nitrogen in different experiments. Results were expressed as q_{O_2} = µl. O_2/mg wet wt./hr.

Tissue weights. At the end of some of the Warburg experiments the diaphragm slips were removed from the flasks, homogenized and dried to constant weight on sand at 90° C.

Cytochrome c. At the end of other Warburg experiments the diaphragm slips were used for the estimation of cytochrome c by the spectrophotometric method of Rosenthal & Drabkin (1943).

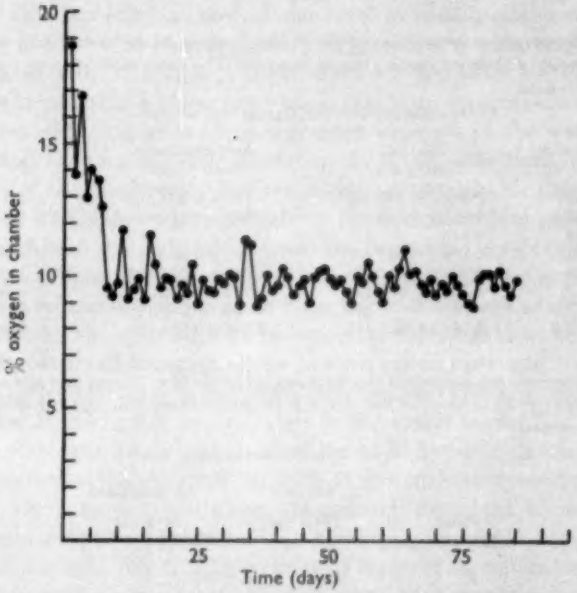

Fig. 2. Mean daily oxygen content of the gas mixture inside the low-oxygen chamber; series *A*.

RESULTS

Blood analysis. The results showed the expected rise in haemoglobin from the control value of 100·3 % (S.E. ± 2·9), = 14·8 g haemoglobin/100 ml. blood, to 154·3 % (S.E. ± 10·7), = 22·3 g haemoglobin/100 ml. blood, in the acclimatized rats (P = < 0·001). Packed cell volume rose from 45·4 % (S.E. ± 2·1) to 59·9 % (S.E. ± 5·2) during acclimatization, 10 weeks–10 months (P = < 0·001).

Tissue respiration. In every experiment, at all levels of oxygen studied *in vitro* the q_{O_2} was lower in the diaphragm from the acclimatized rat than in its control, measured under identical conditions at the same time. Table 1 shows the mean q_{O_2} values for the test and control group at each

oxygen level. It can be seen that the mean q_{O_2} is lower in the acclimatized group than in the controls at all the oxygen concentrations tested. The difference between these means is only statistically significant (Student's t test) at 12, 10 and 5 % oxygen. In two groups, those at 100 and 5 % O_2, where the number of experiments allowed for such statistical treatment, the mean of the difference between the q_{O_2} of the test and control tissue in each experiment was significantly different from zero ($P = < 0{\cdot}001$ at 100 % O_2; $P = < 0{\cdot}001$ at 5 % O_2).

TABLE 1. The oxygen consumption of diaphragm muscle from control and acclimatized rats. The number of observations is given in brackets. The value of P refers to the difference between the means

In vitro O_2 content (% v/v)	O_2 consumption (μl. O_2/mg wet wt./hr) Control Mean ± S.E.	Acclimatized Mean ± S.E.	P
100	1·338 ± 0·218 (6)	1·150 ± 0·208 (6)	> 0·1
21	0·640 ± 0·144 (3)	0·566 ± 0·110 (3)	> 0·5
14	0·450 (1)	0·404 (1)	
12	0·350 ± 0·007 (3)	0·297 ± 0·015 (3)	< 0·05
10	0·294 ± 0·015 (3)	0·245 ± 0·017 (3)	< 0·05
5	0·298 ± 0·024 (7)	0·249 ± 0·032 (7)	< 0·001
3·3	0·257 (1)	0·184 (1)	
2·3	0·181 ± 0·017 (2)	0·146 ± 0·004 (2)	> 0·2

TABLE 2. Cytochrome c content of the diaphragm muscle from the control and acclimatized rats, estimated by the method of Rosenthal & Drabkin (1943). The number of observations is given in brackets

	Cytochrome c (μg/g wet wt.) Control	Acclimatized
Mean	122·2 (11)	89·3 (14)
S.E.	17·0	16·4
P	< 0·001	

Dry weights. The wet weights of the diaphragm slips used in each Warburg experiment have been added together. These values do not represent the total weight of the organ, since the crura and central tendon were discarded. The mean wet weight of the diaphragms from the control rats was 235·1 mg (S.E. ± 12·1) and that of the diaphragms from the acclimatized rats was 234·3 mg (S.E. ± 21·7). Corresponding dry weights were 55·0 mg (S.E. ± 8·8) in the controls and 55·1 mg (S.E. ± 9·6) in the acclimatized rats. These differences are not significant. The wet:dry wt. ratios were 4·27:1·0 in the controls and 4·25:1·0 in the experimental animals.

Cytochrome c. Table 2 shows the results of the cytochrome c estimations in eleven paired experiments and in three acclimatized rats which were investigated immediately, following accidental death. There is a wide

variation in these results although the differences between the mean values is statistically significant ($P = < 0{\cdot}001$). It must be pointed out that these estimations have been made on tissues previously subjected to 2 hr incubation in the Warburg apparatus.

DISCUSSION

These findings show that in rats acclimatized to these hypoxic conditions there is an adaptation at the cellular level in resting diaphragm muscle, resulting in a lowered consumption of oxygen over a wide range of oxygen pressures *in vitro*. This change is probably not due to an increase in those tissue elements with a lower respiration rate than muscle cells since histological investigation showed no apparent changes in the proportion of muscle and connective-tissue elements. It is probable that only the surface cells of the diaphragm slips are taking up oxygen, for Creese, Scholes & Whalen (1958) showed by a study of the normal resting potentials that oxygen diffusion was only adequate in the superficial layers of the isolated diaphragm. Since it was consistently seen on naked-eye observation that the surface area of the diaphragms from the acclimatized rats was greater than that from the controls, with no change in the mean total weights of the organ, the observed lowering of the oxygen uptake per unit weight could not be explained either by a decrease in the number of cells taking part in the respiration *in vitro*; nor by a change in the water content of the tissue. Possibly when the whole animal is subjected to hypoxia the tissue oxygen pressure is lower than normal, in spite of the circulatory and respiratory changes which have taken place. It seemed important to measure the oxygen consumption *in vitro* at the same pO_2 as would have been encountered *in vivo*. For this reason a wide range of O_2 concentration levels was used *in vitro*, from 2 to 100 %, with particular emphasis on the lower range.

It is of interest that in investigations into specific enzyme systems in tissues isolated from acclimatized rats and mice, Udalov (1956) and Kosmolinskii (1956) showed a decrease in the activity of succinic dehydrogenase and cytochrome oxidase, the degree and trend of these changes depending on the length of time and severity of the exposure. Tappan, Baltazar, Potter & Hurtado (1957) also concluded that some alterations occurred at some stage in the succinoxidase system in tissues from guinea-pigs reared at 14,000 ft. (4266 m), but that all components of complex enzyme systems were not influenced equally by the adaptation process. Their results for diaphragm showed a fall in oxidase activity. Ullrick, Whitehorn, Brennan & Krone (1956) measured the oxygen consumption of many tissues taken from rats acclimatized for an average of eleven

weeks to 18,000 ft. (5486 m) and reported no alteration in heart, brain, small intestine, liver, skeletal muscle and diaphragm at 100, 21 and 2 % O_2 *in vitro*, metabolizing glucose; although the adrenal q_{O_2} was elevated and that of the kidney depressed. It is possible that the present results, which differ from those of Ullrick *et al.*, are a reflexion either of the longer periods allowed for acclimatization, or of the differences in the substrate and range of pO_2 levels used in the *in vitro* determinations. These authors pointed out that their inability to show a change in oxygen consumption might be attributed to the range of O_2 levels used, since these levels did not cover the range to which the tissues were exposed *in vivo*. Figure 3 relates the mean q_{O_2} values in the present experiments to the pO_2 in the gas phase with which the isolated diaphragms were in contact. The measurements of oxygen uptake of these tissues from both the control and acclimatized rats were made at the same time under identical conditions, utilizing 0·02 M lactate as substrate at 38·0° C, with the Warburg technique. It can be seen that there is an S-shaped curve relating the oxygen uptake to pO_2 in both groups of animals. The oxygen uptake is independent of changes in pO_2 over the range between 5 % O_2 and 12 % O_2 in the gas phase *in vitro*. The curve for the oxygen uptake of the diaphragms from the acclimatized rats is shifted to the right in comparison with the control group, indicating a reduced consumption of O_2 at any given pO_2. The range of tissue pO_2 *in vivo* in the acclimatized rat is not known, but it is in all probability lower than that in the control rat. There is a statistically significant reduction in the mean q_{O_2} values in the acclimatized rats in comparison with the control rats at the lower pO_2 levels.

The reduction in the rate of oxygen consumption in the diaphragm during acclimatization could be brought about either by a direct effect of the lowered pO_2 on the cells, or by hormonal mechanisms, or by a combination of both effects. There is no direct experimental evidence of alterations in hormonal activity in this work, but other workers have shown that thyroid activity is reduced in hypoxia (Barach, Eckman & Molomut, 1941; Van Middlesworth & Berry, 1951) and it has been suggested by Drabkin (1950*b*) that the thyroid influences tissue respiration by controlling the level of cytochrome c in the tissues. Drabkin showed that increased activity of the thyroid caused a rise in the concentration of cytochrome c in regenerating liver of the rat, while hypothyroidism resulted in a decrease in this concentration. The cytochrome c concentration is directly proportional to the oxidase activity, as shown by Drabkin (1950*a*) in many tissues of the rat, and he considered that the cytochrome c might be the limiting factor.

Drabkin (1947) investigated the effects of short exposures (11 days) to hypoxia but could find no change in the cytochrome c concentration in

regenerating liver of rats. However, Vest & Wang (1950) reported a marked decrease in the concentration of cytochrome c in skeletal muscle of rats and guinea-pigs acclimatized for 35–52 days in air at 320 mm Hg, these changes being reversed a month after return to normal sea-level pressures. The results for rat skeletal muscle showed a fall in cytochrome c from 11·3 to 5·5 mg/100 g, which is a larger fall than that reported in this work for the diaphragm from 12·2 to 8·9 mg/100 g. Vest & Wang considered this decrease might have been brought about by reduced thyroid activity.

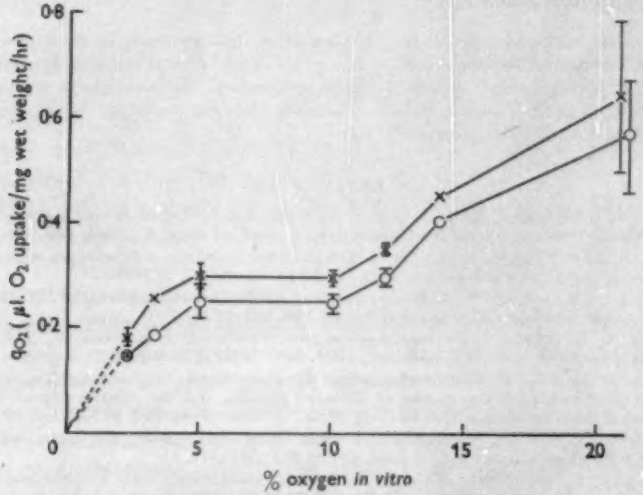

Fig. 3. The relationship between the mean oxygen uptake and the oxygen content of the gas phase *in vitro*, of the isolated diaphragms from control rats (×) and acclimatized rats (○). Vertical bars indicate S.E. of the mean.

If the results reported here for the rat diaphragm are considered to be part of a generalized tissue response to hypoxia, it is possible that the process of acclimatization involves changes at the cellular level resulting in a lowered requirement for oxygen by the whole animal and hence a greater chance of its survival under these conditions.

SUMMARY

1. Rats were acclimatized to low oxygen contents (9–10%) in the inspired air for from 10 weeks to 10 months, at normal atmospheric pressure. Control rats were kept under normal laboratory conditions.

2. Mean haemoglobin values rose from an average of 14·8 g/100 ml. in the controls to 22·8 g/100 ml. in the acclimatized rats. Haematocrit values rose from 45·4 to 59·9% red cells.

3. The O_2 consumption of the isolated resting diaphragms was estimated by the Warburg technique under identical conditions in paired experiments, using lactate as substrate at eight different O_2 levels in the gas phase *in vitro*. At all pO_2 the mean q_{O_2} value was lower in the muscle from the acclimatized rat than in that from the control rat.

4. The mean cytochrome c content of the diaphragms was reduced from 122·2 to 89·3 μg/g wet wt. in the acclimatized rats.

5. There was no alteration in the water content of the diaphragm during acclimatization.

The author wishes to express her gratitude to the late Professor E. M. Killick for her constant help during her supervision of this work. Mr P. Humphrey, Miss B. Lowers and Mr E. G. Roberts provided valuable technical assistance at various stages of this investigation. The findings reported here have been submitted to the University of London as part of the requirements for the degree of Ph.D.

REFERENCES

Barach, A. L., Eckman, M. & Molomut, N. (1941). Modification of resistance to anoxia, with especial reference to high altitude flying. *Amer. J. med. Sci.* **202**, 336–341.

Bruce, H. M. & Parkes, A. S. (1949). Feeding and breeding of laboratory animals; IX, complete cubed diet for mice and rats. *J. Hyg., Camb.*, **47**, 202–208.

Creese, R., Scholes, N. W. & Whalen, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

Drabkin, D. L. (1947). Liver regeneration and cytochrome c metabolism. Influence of anoxia and of injection of cytochrome c. *J. biol. Chem.* **171**, 409–417.

Drabkin, D. L. (1950*a*). The distribution of the chromoproteins, haemoglobin, myoglobin and cytochrome c, in the tissues of different species, and the relationship of the total content of each chromoprotein to body mass. *J. biol. Chem.* **182**, 317–333.

Drabkin, D. L. (1950*b*). Cytochrome c metabolism and liver regeneration; influence of thyroid gland and thyroxine. *J. biol. Chem.* **182**, 335–349.

Duckworth, M. W. (1960). Changes in oxygen consumption and cytochrome c in the diaphragm of acclimatized rats. *J. Physiol.* **152**, 55–56*P*.

Hill, J. R. (1959). The oxygen consumption of new-born and adult mammals. Its dependence on the oxygen tension in the inspired air and on the environmental temperature. *J. Physiol.* **149**, 346–373.

Kerly, M. & Ottaway, J. H. (1954). The effect of diet on the metabolism of glucose and of acetate by rat diaphragm muscle. *J. Physiol.* **123**, 534–552.

Kosmolinskii, F. P. (1956). Effect of thiamine on tissue respiration in hypoxia. *Vop. Pitan.* **15**, 73–75. Cited in *Int. Brit. Abstr. biol. Sci.*, 1959, **13**, 61.

Rosenthal, O. & Drabkin, D. L. (1943). Spectrophotometric studies. IX. The direct micro spectrophotometric determination of cytochrome c. *J. biol. Chem.* **149**, 437–450.

Stickney, J. C. & Van Liere, E. J. (1953). Acclimatization to low oxygen tension. *Physiol. Rev.* **33**, 13–34.

Tappan, D. V., Baltazar, R. D., Potter, V. R. & Hubtado, A. (1957). Alterations in enzymes and metabolites resulting from adaptation to low oxygen tensions. *Amer. J. Physiol.* **190**, 93–98.

Udalov, I. F. (1956). Mechanism of action of group B vitamins in conditions of insufficient oxygen. *Vop. Pitan.* **15**, 22–26. Cited in *Int. Brit. Abstr. biol. Sci.*, 1959, **13**, 61.

Ullrick, W. C., Whitehorn, W. V., Brennan, B. B. & Krone, J. G. (1956). Tissue respiration of rats acclimatized to low barometric pressure. *J. appl. Physiol.* **9**, 49–52.

Van Middlesworth, L. & Berry, M. M. (1951). Iodide metabolism during anoxia, nephrectomy, trauma, avitaminoses and starvation in the rat. *Amer. J. Physiol.* **167**, 576–580.

Vest, M. & Wang, S. J. (1950). Veränderung des Cytochrom-c-Gehaltes der Muskulatur in grossen Höhen. *Helv. physiol. acta*, **8**, 180–185.

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A GROUP OF NEURONES IN THE DORSAL HORN ASSOCIATED WITH CUTANEOUS MECHANORECEPTORS

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(*Received* 11 *January* 1961)

Slow potential changes, resulting from stimulation of cutaneous and muscle afferent nerve fibres, can be recorded from spinal cords extracellularly through glass capillary micro-electrodes (Eccles, Fatt, Landgren & Winsbury, 1954; Coombs, Curtis & Landgren, 1956; Fernandez de Molina & Gray, 1957; Bravo & Fernandez de Molina, 1960). These potentials are a resultant of the activity of all the cells in the neighbourhood of the electrode tip and may be termed mass responses. Fernandez de Molina & Gray (1957) recognized three phases of the mass response produced on electrical stimulation of the sural and the saphenous nerves in the cat, each phase having a characteristic time course and distribution in the cord. Analysis of their properties led the authors to attribute all the phases to activity in post-synaptic elements and to suggest that synchronous synaptic potentials made a major contribution.

The experiments described in the present paper were done on cats. The activity of single spinal units, which could be excited both by electrical stimulation of the medial plantar nerve and by mechanical stimulation of the foot, was recorded through micro-electrodes; when electrical stimulation was used the impulses were seen superimposed on the mass response. The properties of spinal interneurones have been studied by several workers using intracellular micro-electrodes; under such conditions certain properties of the impaled cell may be accurately determined (Woodbury & Patton, 1952; Frank & Fuortes, 1955, 1956; Haapanan, Kolmodin & Skoglund, 1958; Hunt & Kuno, 1959*a*, *b*). In the present experiments the activity of the individual cell was superimposed on a potential change which reflected the electrical activity of a whole population of cells; it was therefore possible to relate the discharge of a single unit to the activity of the total population and to analyse the properties of groups of cells from consideration of both single unit recordings and mass responses. This work was done during the course of an investigation into the interaction

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of primary signals at synaptic levels in the cord and consequently activity was recorded from only about 150 units, of which 75 were suitable for analysis. The purpose of the analysis was to begin the specification of interneurones, particularly second-order neurones, associated with mechanoreceptors in the pad. The work deals with the input to the interneurones studied and the properties of their discharge, but does not take into account the destination of their axons. More information on the latter aspect of the problem has been obtained by Dr R. M. Eccles.

A preliminary account has appeared elsewhere (Armett, Gray & Palmer, 1958).

METHODS

Most experiments were performed on cats anaesthetized with chloralose (0·05 g/kg) and urethane (0·5 g/kg), injected intravenously after induction with ethyl chloride and ether. In a few experiments the dissection was done under sodium pentathone anaesthesia, after which the animal was decerebrated and the anaesthetic allowed to wear off. The spinal cord was exposed from S1 to L5 and divided in the thorax; the dura was also divided in order to reduce the movements of the cord due to respiratory activity. The medial plantar nerve was exposed for electrical stimulation but was not divided. The preparation was then mounted rigidly in the manner described by Fernandez de Molina & Gray (1957). The spinal cord was protected by a paraffin pool. The medial plantar nerve was placed over a pair of platinum electrodes; the nerve and electrodes were buried in paraffin wax (m.p. 39° C).

Pulses of about 100 μsec duration from a low impedance source were applied to the nerve through the platinum electrodes.

The spinal cord potentials were recorded through glass capillary micro-electrodes of tip diameter *ca.* 1·0 μ filled with NaCl solution 10 g/100 ml. Electrodes of resistance greater than 5 MΩ were rejected. During an experiment the electrode was held in position by a manipulator; this was graduated along three axes the angles between which were variable; adjustments to about 50 μ were thus effected. There was also a fine downward movement operated on a hydraulic principle and this could provide movements down to the order of 1 μ. The electrode was connected to a high-impedance recording system by means of a Ag-AgCl contact. The indifferent electrode, a Ag-AgCl-NaCl (in agar, and cotton wool) system, was inserted between the skin and the back muscles of the cat and connected to the other side of the recording system. The animal and the holding frame were earthed independently. The potentials were amplified with an instrument of suitable band-width, and displayed on one beam of a cathode-ray oscilloscope.

In some experiments the dorsal root volley was recorded. A filament of the dorsal root was cut as near to the spinal cord as possible and its peripheral end placed over a pair of platinum electrodes; the potentials were amplified and displayed on the second beam of the oscilloscope.

Procedure. Those segments of the spinal cord activated by electrical stimulation of the medial plantar nerve were located in the manner described by Fernandez de Molina & Gray (1957). The afferent volley was recorded at points along the length of the cord through a micro-electrode placed over the dorsal root entry zone, and the region of the cord opposite the rootlet that gave the maximum evoked response was judged to be that containing most activity. Two distinct elevations were usually seen in the primary fibre potentials but the maxima were found within 2 mm of each other. The pia was opened in the active region and the micro-electrode lowered on to the mid line of the cord. The co-ordinates were noted and tracks were then made into the cord at intervals not less than 0·1 mm apart in both head-to-tail and side-to-side axis. In each track a record of the mass response was taken routinely

(*a*) 1 mm below the surface of the cord, (*b*) as near as possible to the position of maximum amplitude of the main early response (phase II in the terminology of Fernandez de Molina & Gray (1957)), and (*c*) at the point where the sign of the phase II potential reversed. The co-ordinates of these points and of any single unit investigated were recorded. At the end of a series, the electrode was left in the cord and the positions of the units were identified by the histological techniques described in a previous paper (Fernandez de Molina & Gray, 1957).

RESULTS

General

The mass responses recorded through micro-electrodes in the dorsal horn on stimulation of the medial plantar nerve were similar to those described by Coombs *et al.* (1956) and Fernandez de Molina & Gray (1957). The main early response (phase II in the terminology of Fernandez de Molina & Gray) had a maximum in a position medial to that of the equivalent potential on sural stimulation; the small early phase I was not investigated.

Fernandez de Molina & Gray concluded that the phase II potential was the mass response of a population of neurones, at least some of which were monosynaptically connected with the lowest-threshold afferent fibres from the skin. Since the primary object of the present experiments was to find units in the spinal cord that were second-order to mechanoreceptors in the pad, the region searched was that in which the main early phase (phase II) of the mass response was large. The units considered are therefore not, and were not intended to be, a random sample of interneurones with cutaneous connexions. However, all units which were found in this region and which could be excited both by electrical stimulation of the medial plantar nerve and by mechanical stimulation of the foot are included in the analysis.

The activity of the single units was seen as a spike discharge superimposed on a mass response (Fig. 1). The latency of the peak of the first impulse of the discharge was measured from the negative peak of the incoming volley, and the values obtained in the anaesthetized preparations have been plotted as a frequency histogram in Fig. 2. The histogram shows two peaks, at 1 and at 3 msec, and a dip at about 2 msec. In certain experiments recordings were made from a dorsal rootlet (Fig. 1 top beam) and the area of the primary fibre volley could then be used as a measure of the threshold for a response from the unit. The relation between the area of the incoming volley at threshold and the latency of the first impulse (measured under suprathreshold conditions) is shown for 25 units in Fig. 3. The units in the earlier hump of the latency distribution (Fig. 2), that is units with a latency less than 2 msec, all had low thresholds (see Fig. 1*a*). The other units, with one exception, had higher thresholds. The units

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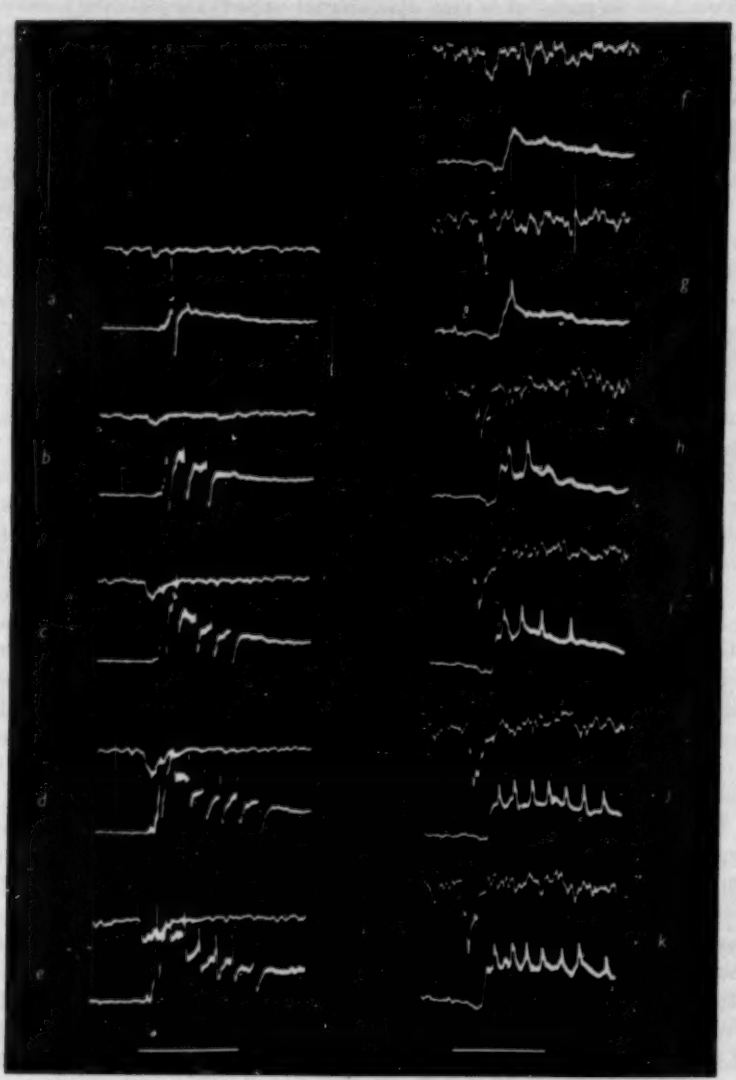

Fig. 1. Effect of increasing the strength of stimulus applied to the medial plantar nerve on the response of a single unit in the dorsal horn. Upper beam, record from dorsal rootlet; lower beam, record from unit in the grey matter. *a–e*, effect of increasing stimulus strength on a unit responding at a short latency. *f–k*, a comparable set of records for a unit responding at a longer latency. Negative deflexions in this and all subsequent records are upwards. The horizontal bar represents 10 msec.

with low thresholds and short latencies appear to form some sort of a group and their properties are considered in the next section.

Short-latency units

The mean value of the latency of these units in anaesthetized animals was 1·1 msec (standard deviation 0·34 msec, number 35). A few units were isolated in decerebrate animals and these had significantly shorter latencies (mean 0·76 msec, S.D. 0·22 msec, number 10).

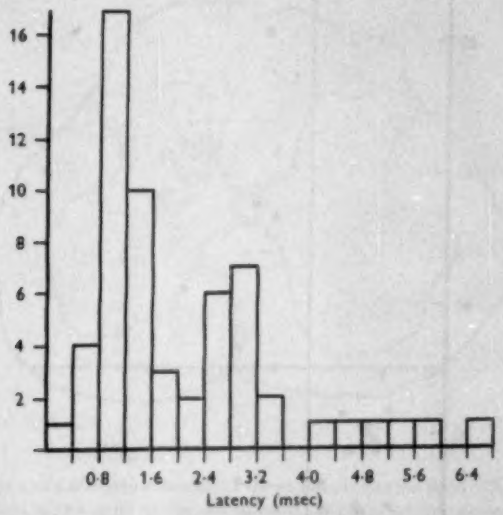

Fig. 2. Frequency distribution of latency of all units isolated from anaesthetized animals and analysed. Latency (msec) from negative peak of incoming volley to the peak of the first impulse, with stimulus strength much greater than threshold.

All the units in this group, unlike the remainder, were recorded from positions at which the main early phase (phase II) of the mass response was negative. There was in fact a close relation between the distribution of the short-latency units and the peak of the early phase of the mass response. This was shown quantitatively in some experiments. The distance of the units from the point on the track where the early phase reversed was measured along a dorsoventral line; also in each track the peak of this early mass response was related to the reversal point. The mean position of the units was 0·73 mm dorsal to the reversal point and they were scattered with a standard deviation of 0·25 mm (number of observations 14). The position of the peak was in the mean 0·59 mm (S.D. 0·23 mm, number 15) dorsal to the reversal point. The anatomical positions of some

of the units in the short-latency group are shown as full circles in Fig. 4. These positions were determined by a co-ordinate method and the points have been transferred to an equivalent position on one diagram. The method is liable to error for individual points, but is likely to give a satisfactory picture of the general distribution.

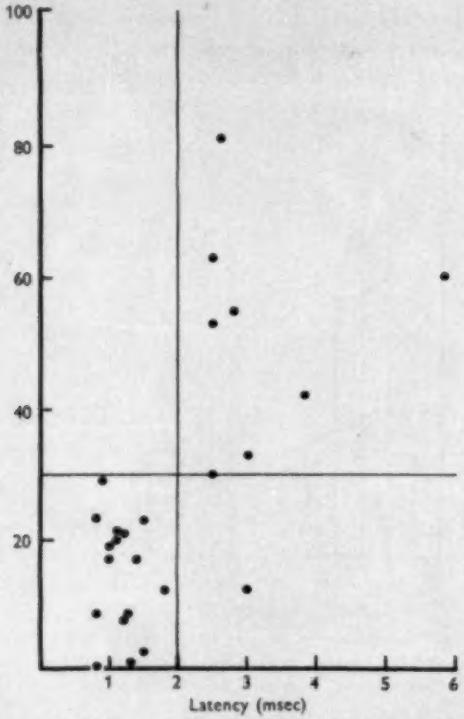

Fig. 3. Latency and threshold scatter diagram. Ordinate, area of the dorsal root volley at threshold, given as a percentage of the maximal area; abscissa, latency.

A repetitive discharge usually resulted from a single stimulus (Fig. 1*a–e*). A frequency distribution of the latencies of all the impulses in the responses to single maximal shocks of all the units was constructed. This temporal distribution of impulse activity was found to be consistent with the time course of the mass response. It showed a break at approximately 3 msec, which is about the time that the later (phase III) part of the mass response breaks the continuity of the falling phase of the earlier part. A break in the sequence of the impulse discharge from individual units has been seen quite frequently; for example, in Fig. 1*c–e* the interval between the

second and third impulses is longer than either the preceding or succeeding intervals. Figure 5 illustrates a particularly clear example of this and shows how regular the gap is for a particular unit. Figure 5*a* shows the pattern of discharge in response to ten different electrical stimuli of approximately equal strength. This pattern is analysed in Fig. 5*b*, in which the preceding interspike interval is plotted against the total time to the spike from the incoming volley in the primary receptor fibres. The third spike (×) occurred consistently at an interval which was appreciably

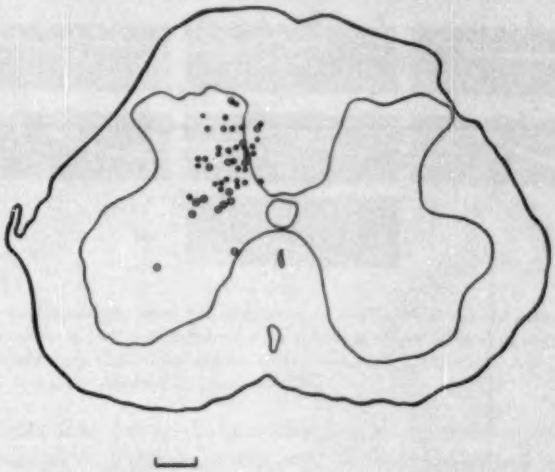

Fig. 4. Diagram of a transverse section of the spinal cord at the level of L 6, to show the distribution of the units in the dorsal horn. ● short-latency units; ○, long-latency units. Points have been found by a co-ordinate method and then transposed to corresponding positions on this standard diagram of the cord; the positions of individual points should therefore be regarded with reserve. Horizontal bar represents 0·5 mm.

longer than the preceding interval and, in about half the trials, longer than subsequent intervals. In this particular experiment there seemed to be a high probability of a fourth (●) or fifth spike (□) occurring at about 8 msec. Figure 5 may be compared with certain figures shown by Hunt & Kuno (1959*b*). The precise timing and significance of these irregularities in the discharge pattern need more investigation; a few internal recordings obtained incidentally by R. M. Eccles and ourselves do, however, show that the synaptic activity of a single cell may show phases of activity, which could well be associated with the discharge patterns observed. Figure 6 shows records obtained internally: in *a*–*e* the size of the synaptic activity is of the right order of magnitude, although the impulse is very

small and slow, indicating a low membrane potential; *f–g* were taken later and the cell was then in very poor condition, although it was still able to fire an impulse. *a* is a record of spontaneous activity and probably

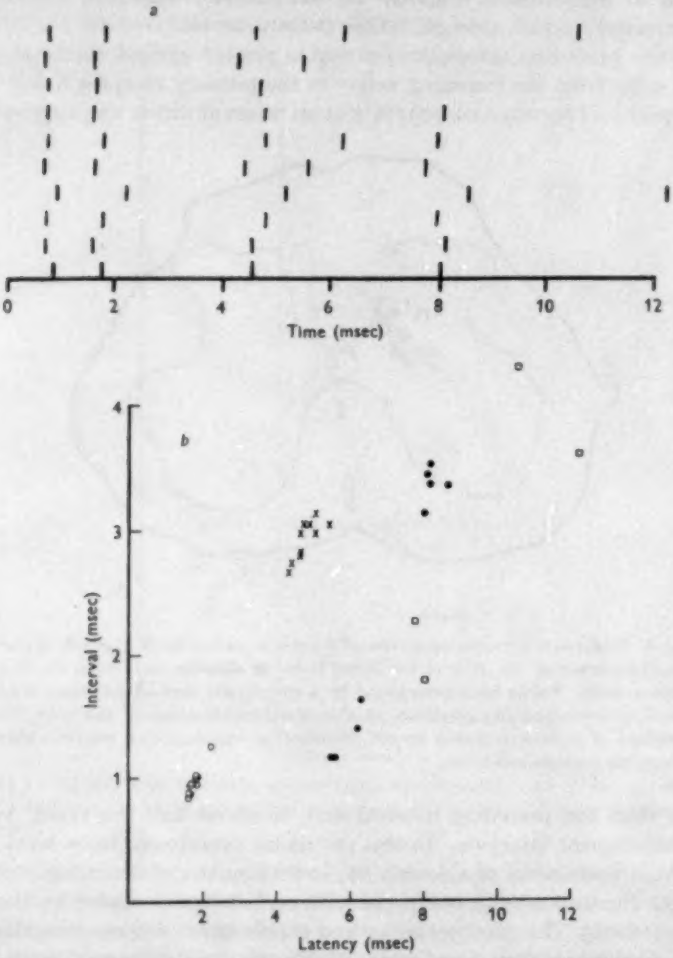

Fig. 5. *a*, diagram to illustrate the pattern of impulse discharge in a single unit. Each horizontal row indicates the time pattern of the impulses resulting from a single shock. The response patterns to ten separate shocks are shown. *b*, the same data as *a* plotted as a graph. Ordinate, interval between a particular spike and the preceding spike; abscissa, time from the incoming volley to the spike. The position of each spike in its own discharge is indicated by the symbol: ○ 2nd impulse, × 3rd impulse, ● 4th impulse, □ 5th impulse.

represents synaptic responses to single presynaptic impulses. *b* and *c* were obtained with very small electrical stimuli and are essentially the same, but for the amplification; in these there is a suggestion of a second deflexion that is more than the random background. In *d*, with a bigger stimulus, a second deflexion is clear; *e* was above but near threshold. *f* and *g* are included to show the effects of big stimuli and are recorded on a slower sweep. With stimulus strengths increasing above the threshold value the later activity increases and the peak of the later hump gets progressively later.

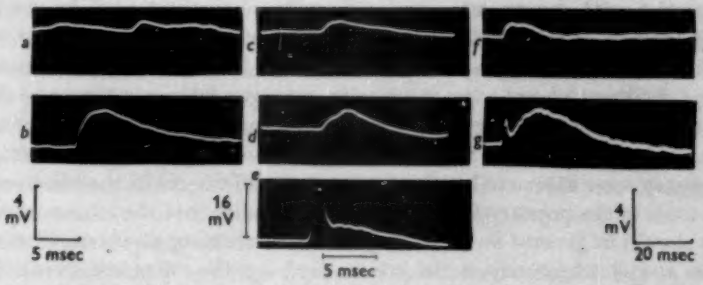

Fig. 6. Intracellularly recorded responses of a cell of the short latency type: *a*, spontaneous activity; *b*, response to a stimulus of low intensity; *c–e*, stimulus strength increased until threshold for spike reached; *f–g*, slow time trace; in *g* the stimulus was many times the threshold value.

Of the units analysed in the short-latency group, 45% were excited by light mechanical stimulation of the pad. Most of the others were excited by movements of the hairs around the pads; a few had receptive fields on the heel or dorsum of the foot, and two required much bigger forces applied to the whole foot for excitation.

Longer-latency units

Those found are referred to mainly for purposes of contrast with the group just considered. The thresholds of the units were higher than those of the short-latency group; nevertheless, a response could usually be obtained before the main early peak of the dorsal root volley reached a maximum (Fig. 1*g*).

The position in the cord of these units was not related to the position of the early phase of the mass response, in fact 46% of those recorded were found when the early mass response was positive (Fig. 1*f–k*). The anatomical positions of a sample of these units are shown as open circles in Fig. 4. There is no reason to regard these longer-latency units as a single group; the positions in which the units were found might even suggest the contrary.

DISCUSSION

In order to determine the function of cells in the spinal cord their properties and connexions must be so far determined that it is possible to work on a particular and sufficiently homogeneous group of cells. The purpose of the analysis presented here was to identify a cell group connected mono-synaptically to low-threshold mechanoreceptors in the skin of the foot, and to define some of the properties of the cells.

The units analysed were divided into a short-latency group and a remainder with longer latencies. All units whose responses lay in the short-latency distribution could be fired when the incoming volley was confined to a small proportion of the low-threshold, fast-conducting receptor fibres, whereas the units with a longer latency were only fired when the incoming volley was bigger. Distinctions due to latency and threshold might well be reflexions of the same process if the differences in latency were associated with great enough differences in the conduction velocities of the primary fibres. This would require that the longer-latency unit should in general be excited by fibres conducting at about 40 m/sec, while the short-latency units are excited by the 60 m/sec group (see Fernandez de Molina & Gray, 1957). Longer-latency units could often be excited by stimuli which were not maximal for the faster group. It would seem therefore that, in part at least, the two parameters, latency and threshold, can be regarded as independent.

The spatial distribution of the short-latency units has been given in both electrical and anatomical terms. In electrical terms the units are entirely within the range in which the main early phase of the mass response (phase II potential) is negative and relatively large, the distribution showing a maximum which was not significantly different from the position of the peak of the phase II potential; the anatomical distribution is also similar to that of the phase II potential (but is medial to that found by Coombs *et al.* (1956) and Fernandez de Molina & Gray (1957) for sural responses). The time pattern of the impulses suggests that the phase II potentials are the resultant of early activity in the cells of the short-latency group. The latency of the response, the minimum stimulus required to excite it, and the spatial and temporal distributions of the activity, therefore, all relate the short-latency units to the phase II potential. The spatial and temporal properties of these cells are similar to those of a group of cells identified by Wall (1960) in the dorsal horn of the cat.

When the preparations were not under the influence of an anaesthetic, the average latency of the first impulse in the responses of the short latency cells was 0·76 msec (S.D. 0·22 msec). This figures suggests that some cells of this group must be monosynaptically connected to the

receptor neurones, and if these cells do constitute a related group it seems likely that the cells are all monosynaptically connected with the fastest conducting fibres from the skin. The pattern of the impulse discharge in some experiments, and the intracellularly recorded synaptic potential in others, showed a discontinuity which suggests that the initial phase of monosynaptic activation is followed by later polysynaptic activity; both components can be excited by low-amplitude mechanical stimulation of the skin (C. J. Armett, J. A. B. Gray, R. W. Hunspeiger & S. Lal unpublished) and by electrical stimuli, which are submaximal for the fastest fibres.

In these experiments the cells analysed were all activated by mechanical stimulation of the foot, most of them by small localized displacements of the skin or hairs. The thermal and chemical sensitivities were not tested. It is possible however that these cells, like those analysed by Wall (1960), could be activated by such stimuli. The primary units which are the most sensitive to thermal and chemical events are small, and the evidence presented here and by Fernandez de Molina & Gray (1957) would suggest that these fibres are not necessary to the full development of the early phase of the response of these cells.

These results help to define a group of spinal cord cells not only in terms of their anatomical position but more particularly in relation to the mass responses of the cells in the dorsal horn. Properties of the units have previously been deduced from a study of their mass responses (Coombs *et al.* 1956; Fernandez de Molina & Gray, 1957) and the present experiments confirm suggestions that the cells are second-order to the fastest-conducting afferent fibres, many of which connect with highly sensitive mechano-receptors in the foot. Neither these results nor those of Wall (1960) tell us anything about the destination of the axons of the cells. Later evidence, however (R. M. Eccles, unpublished), suggests that the cells of this group may be further classified according to the connexions of their axons.

The long-latency units that were found during the course of this study could clearly contribute to the later part of the mass response (phase III). The units were, however, widely scattered and do not seem to represent a homogeneous group. The evidence presented here has shown that the late phase of the mass response is likewise of mixed origin. It includes not only the activity of the long-latency units but also the polysynaptic activity of the short-latency units, and probably activity of yet other types of cell. Further investigation into the properties of this phase of the mass response is unlikely to yield more information concerning cell function in the cord.

SUMMARY

1. Mass responses and single-unit activity produced in the dorsal horn of the cat's spinal cord by electrical stimulation of the medial plantar

nerve were recorded extracellularly through micro-electrodes. An analysis of the activity was made only for those single units that could respond also to mechanical stimulation of the foot.

2. The components of the mass responses were similar to those recorded on sural stimulation; the main negative deflexion (phase II of Fernandez de Molina & Gray, 1957) was, however, found medial to that of the equivalent phase produced on sural stimulation.

3. The single units analysed could be divided into a group that responded at short latencies from the incoming volley when low-threshold afferent fibres were stimulated, and a remainder that responded at longer latencies and to higher stimulus strengths.

4. The spatial and temporal distributions of the activity of single units were examined.

5. It is concluded that the short-latency cells are monosynaptically connected to fast-conducting fibres from mechanoreceptors and that an early phase of their activity gives rise to the main early negative phase of the mass response (phase II); a later phase of the impulse discharge is polysynaptic in origin and is thought to contribute to the later negative phase of the mass response (phase III).

One of us (C.J.A.) was supported by a grant from the Medical Research Council.

REFERENCES

ARMETT, C. J., GRAY, J. A. B. & PALMER, J. F. (1958). Properties of neurones in the dorsal horn of the spinal cord of the cat. *J. Physiol.* **145**, 49–50*P*.

BRAVO, M. C. & FERNANDEZ DE MOLINA, A. (1960). Frog's spinal cord potentials generated by impulses in cutaneous afferent fibres. *J. Physiol.* **152**, 14–15*P*.

COOMBS, J. S., CURTIS, D. R. & LANDGREN, S. (1956). Spinal cord potentials generated by impulses in muscle and cutaneous afferent fibres. *J. Neurophysiol.* **19**, 452–468.

ECCLES, J. C., FATT, P., LANDGREN, S. & WINSBURY, G. J. (1954). Spinal cord potentials generated by volleys in large muscle afferents. *J. Physiol.* **125**, 590–606.

FERNANDEZ DE MOLINA, A. & GRAY, J. A. B. (1957). Activity in the dorsal spinal grey matter after stimulation of cutaneous nerves. *J. Physiol.* **137**, 126–140.

FRANK, K. & FUORTES, M. G. F. (1955). Potentials recorded from the spinal cord with microelectrodes. *J. Physiol.* **130**, 625–654.

FRANK, K. & FUORTES, M. G. F. (1956). Unitary activity of spinal interneurones of cats. *J. Physiol.* **131**, 424–435.

HAAPANAN, L., KOLMODIN, G. M. & SKOGLUND, C. R. (1958). Membrane and action potentials of spinal interneurones in the cat. *Acta physiol. scand.* **43**, 315–348.

HUNT, C. C. & KUNO, M. (1959*a*). Properties of spinal interneurones. *J. Physiol.* **147**, 346–363.

HUNT, C. C. & KUNO, M. (1959*b*). Background discharge and evoked responses of spinal interneurones. *J. Physiol.* **147**, 364–384.

WALL, P. D. (1960). Cord cells responding to touch, damage, and temperature of skin. *J. Neurophysiol.* **23**, 197–210.

WOODBURY, J. W. & PATTON, H. D. (1952). Electrical activity of single spinal cord elements. *Cold Spr. Harb. Symp. quant. Biol.* **17**, 185–188.

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INTERNAL CHLORIDE CONCENTRATION AND CHLORIDE EFFLUX OF FROG MUSCLE

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It has recently been shown that the chloride conductance of the inactive muscle membrane is about twice the potassium conductance (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). It is of interest to know whether the flux of chloride is large enough to account for the high conductance, assuming that the inward and outward movement of chloride are independent. Keynes (1954) concluded that the potassium fluxes were not independent because, on that assumption, the potassium flux was too small to contribute substantially to the membrane conductance. It is known that the presence of a foreign anion in the external solution slows the efflux of ^{36}Cl (Harris, 1958), but that nitrate and iodide do not influence the potassium flux (Edwards, Harris & Nishie, 1957). The experiments in this paper were designed to measure the internal chloride concentration and the chloride efflux. In some experiments a foreign anion was used to slow the efflux of ^{36}Cl and allow a clearer separation of intracellular and extracellular chloride.

METHODS

All experiments were done on the sartorius muscle of English frogs (*Rana temporaria*) at room temperature (15–20° C). The muscle was cut from its pelvic attachment and fine threads were tied to the pelvic and tibial tendons. The pelvic tendon was cleaned by cutting away the fibres of other muscles. The sartorius was tied to a frame made of thin glass rod so that both sides of the muscle were exposed to the solution, and the rod was moved up and down by a small electric motor to ensure adequate stirring.

Solutions. The composition of the solutions used is given in Table 1. The normal Ringer's fluid had the same composition as that described previously (Adrian, 1956), and was made up in the same way. Nitrate was substituted for chloride by using $NaNO_3$ and KNO_3 in place of NaCl and KCl. The NO_3-Ringer's fluid contained 3·6 mM-Cl as $CaCl_2$. When the external KCl concentration was raised the NaCl concentration was left unaltered, so that at equilibrium the muscle fibres would have the same volume as in Ringer's fluid.

Small quantities (2–3 ml.) of solutions containing tracer chloride were made up from 1 M solutions of $Na^{36}Cl$ and $K^{36}Cl$ supplied by A.E.R.E. Harwell. Since the volume of the loading solution was only about 40 times the volume of a single sartorius muscle, the specific activity could not be considered constant from experiment to experiment. The specific activity was measured after each muscle had been soaked in the radioactive solution. When muscles were loaded in solutions with a high KCl concentration, both the specific activity and the concentrations of potassium and chloride were altered, because the muscle

fibres removed potassium and chloride from the loading solution without an equivalent quantity of water. The variation in the external potassium concentration of the loading solution was not greater than 10%.

Measurement of radioactivity. The efflux of radioactivity from the muscles was followed by suspending them in a series of tubes containing 1 ml. of inactive solution, and measuring the radioactivity emerging from the muscle in a measured time. At the end of the experiment the dried muscle was extracted in distilled water to determine the radioactivity remaining in the muscle (Adrian, 1960).

The long half-life of ^{36}Cl (4×10^5 years) eliminates any problems due to the decay of the isotope, but it limits the available specific activity. Furthermore, the low energy of the particles which it emits makes it unsuitable for counting in conventional liquid counters. Reasonably high counting efficiency was obtained by precipitating the ^{36}Cl and inactive Cl as AgCl and counting the precipitate with a mica end-window G–M tube (1·7–2·3 mg/cm^2). Because self absorption in the precipitate could not be eliminated it was important that each sample of AgCl should contain the same amount of chloride. When the loss of radioactivity took place into a solution containing nitrate, 1 ml. of ordinary Ringer's fluid was added before the precipitation of the chloride. The AgCl precipitates, after coagulation by boiling, were centrifuged and resuspended in a 1:1 mixture of glycerol and ethanol. The suspension was then transferred to a filter-paper disk, the suspending fluid being removed by suction to leave an evenly spread deposit of AgCl. By waxing the outer ring of the disk (diam. 2·4 cm), the precipitate was confined to a circular area in the centre (diam. 1·3 cm).

Table 1. Composition of solutions

	K^+	Na^+	Ca^{2+}	Cl^-	NO_3^-	SCN^-	HPO_4^{2-}	$H_2PO_4^-$
	(mg ion/l. solution)							
Cl-Ringer's fluid	2·5	120	1·8	121·1	—	—	2·15	0·85
NO_3-Ringer's fluid	2·5	120	1·8	3·6	117·5	—	2·15	0·85
10% SCN-Ringer's fluid	2·5	120	1·8	109·1	—	12	2·15	0·85
High-KCl solution	50	120	1·8	168·6	—	—	2·15	0·85

Some of the samples, particularly at the end of an experiment, had very low counting rates (about 1 count/min above background) and it was necessary to count them for as long as 14 hr to achieve a S.E. of ±10%. Frequent background counts for comparable periods showed that it was stable (approx. 6 counts/min; exact values differed for different tubes by a small amount).

RESULTS

The efflux of chloride into solutions containing chloride or nitrate

Sartorius muscles were soaked for 2½ hr in a Ringer's fluid of normal composition which had been made up with $Na^{36}Cl$. If the rate at which ^{36}Cl is lost from the muscle into inactive Cl-Ringer's fluid (2·5 mM-K, 120 mM-Cl) is determined, the radioactivity of the muscle at any time can be obtained by adding the radioactivity left in the muscle at the end of the experiment to the radioactivity lost by the muscle in each of the collecting periods after the time in question. If the muscle radioactivity is plotted semilogarithmically against time, the points lie on a straight line for times greater than about 20 min (Fig. 1, open circles). At first sight it would seem reasonable to assume that the linear portion of the

curve represents the loss of intracellular radioactivity. If this were so, extrapolation of the linear part of the curve would give an estimate (subject to certain errors; see below) of the intracellular chloride. Three such experiments gave a mean value of 0·57 m-mole/kg muscle. If this figure represents the total intracellular chloride, muscles soaked for the same length of time in ^{36}Cl-Ringer's fluid and transferred to NO_3-Ringer's

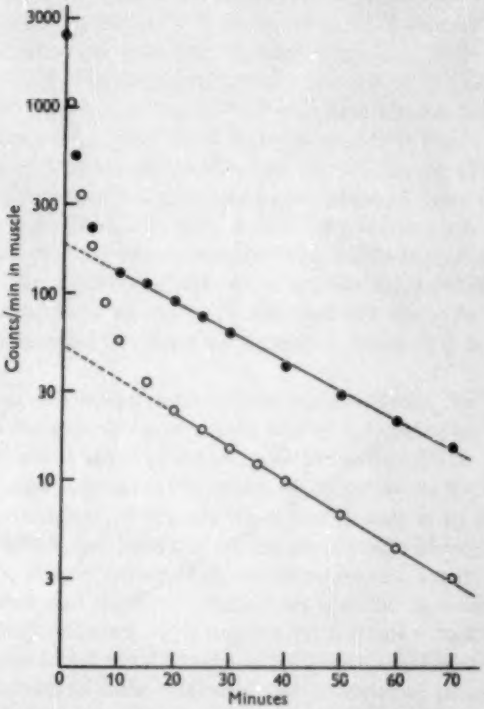

Fig. 1. The loss of ^{36}Cl from two muscles into inactive Cl-Ringer's fluid (○) and inactive NO_3-Ringer's fluid (●). The muscles weighed 38 mg and 42 mg respectively. The specific activity of the soaking solution was 2×10^6 counts/min.m-mole.

fluid or 10% SCN-Ringer's fluid, should have the same initial quantity of ^{36}Cl in the fibres. The curve with filled circles in Fig. 1 shows the radioactivity of a muscle losing ^{36}Cl into NO_3-Ringer's fluid. The two curves in Fig. 1 may be compared directly, because the specific activity of the soaking solution was the same in the two experiments and the two muscles were of nearly equal weights (38 and 42 mg). Contrary to expectation, the linear parts of both curves have nearly the same slope, but their intercepts at zero time are very different. Four such experiments gave a

mean value for the apparent intracellular chloride of 3·12 m-mole/kg muscle. Since both groups of muscles were treated identically up to the moment of transfer to the inactive solution, the slow linear loss of radioactivity in the Cl-Ringer's fluid must represent either a small fraction of the intracellular chloride, possibly from fibres with long time constants,

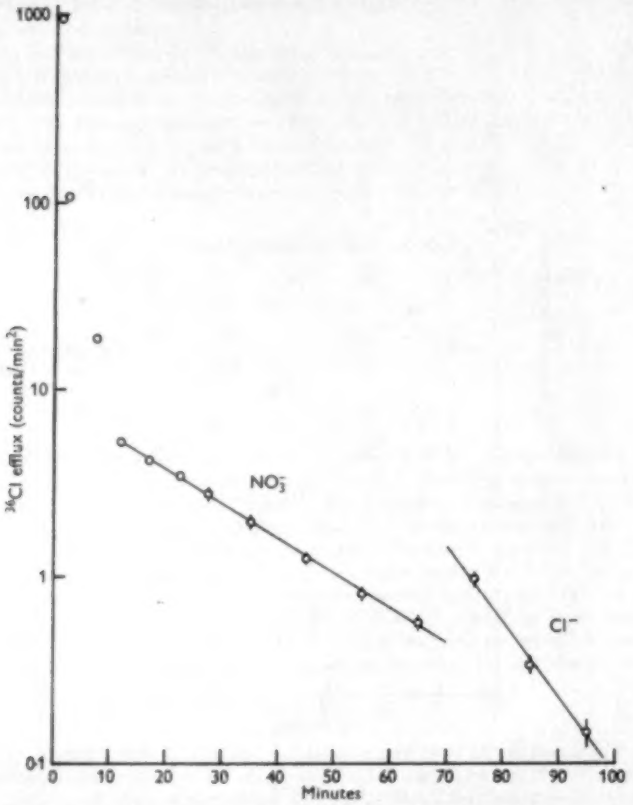

Fig. 2. The rate of loss of ^{36}Cl into NO_3-Ringer's fluid and subsequently into Cl-Ringer's fluid.

or part of the extracellular chloride which exchanges very slowly with the surrounding fluid. Both assumptions imply that the loss of the greater part of the intracellular radioactivity takes place sufficiently rapidly in Cl-Ringer's fluid to make it difficult to distinguish from the loss of ordinary extracellular chloride.

Figure 2 shows the effect of returning a muscle to Cl-Ringer's fluid after

an initial period in NO_3-Ringer's fluid. The rate of loss has been plotted in counts/min². In the Cl-Ringer's fluid the rate of loss of radioactivity is approximately doubled, and the time constant halved. This effect is reversible if the muscle is returned to NO_3-Ringer's fluid. Table 2 summarizes the results of four experiments, including two using a Ringer's fluid with 10% of the chloride replaced by thiocyanate. The transfer of the muscle from NO_3-Ringer's fluid to 10% SCN-Ringer's fluid affects the time constant more than the transfer from 10% SCN-Ringer's fluid to Cl-Ringer's fluid. The time constant for the loss of ^{36}Cl into Cl-Ringer's fluid was about 11 min. A loss of ^{36}Cl with this time constant would be difficult to distinguish in a curve of the type shown in Fig. 1 (open circles). Levi & Ussing (1948) measured the loss of ^{36}Cl into a Cl-Ringer's fluid and were able to distinguish a fast and slow phase of loss. The slow phase was linear between 20 and 80 min, with a time constant of about 17 min. From their data it is uncertain whether this value can be considered the time constant for the loss of the whole of the intracellular chloride or whether it corresponds to the time constant for the slow linear loss of radioactivity observed in these experiments when ^{36}Cl is lost into Cl-Ringer's fluid.

Estimation of the initial intracellular radioactivity, by extrapolation to zero time of the type of curve shown in Fig. 1, leads to an over-estimate because no account is taken of diffusion in the extracellular space. Harris & Burn (1949) and Keynes (1954) have given solutions for the equations governing the exchange of tracers when the muscle is in a steady state (i.e. when the efflux and influx of an ion are equal). However, when ^{36}Cl is lost into NO_3-Ringer's fluid their solutions do not apply because there is a loss of active and inactive chloride, so that the intracellular specific activity remains unaltered (ignoring the very small influx which results from the presence of 3·6 mM-Cl in the NO_3-Ringer's fluid), and the internal chloride concentration falls. Because the membrane potential remains unaltered (see below) it is reasonable to assume that the outward movement of chloride is proportional to the internal concentration. Making this assumption and representing the muscle by a single intracellular compartment in series with a single extracellular compartment, it can be shown that extrapolation of the slow phase of loss will over-estimate the intracellular radioactivity by about 25%. (The time constants for the loss of extracellular and intracellular ^{36}Cl were taken as 2 and 22 min respectively. A correction of 25% is probably somewhat too small, since the two-compartment assumption ignores the fact that the extracellular conditions are not uniform for all distances from the surface of the muscle.)

Extrapolation to zero time in the four experiments of Table 2 gives an average of 3·12 m-mole/kg muscle for the chloride associated with the

slow loss of ^{36}Cl. This chloride might all be intracellular, but if all or part of the ^{36}Cl which is lost slowly into Cl-Ringer's fluid (0·57 m-mole/kg muscle) has an extracellular origin, the intracellular chloride will be less than 3·12 m-mole/kg muscle. Applying a diffusion correction of 25 % to both figures and expressing the chloride concentration in terms of the fibre water (66 % of the muscle wet weight) an upper and lower limit of 3·8 and 3·1 m-mole/kg fibre water are obtained.

TABLE 2. The time constants (min) for the loss of ^{36}Cl into Ringer's fluids with different anion composition

Expt. No.	NO_3^-	SCN^-	Cl^-	$[Cl_i]^*$ (m-mole/kg fibre water)
4	23	—	11	3·9
5	19	—	10	5·1
6	—	15	13	5·5
7	22	15	—	4·2

* $[Cl_i]$ was calculated by extrapolating the slow loss in the first solution to zero time, and is not corrected for extracellular diffusion. In expts. 4, 5, 7, the initial loss of ^{36}Cl took place into NO_3-Ringer's fluid. In expt. 6 the initial loss of ^{36}Cl took place into a Ringer's fluid with 10 % of the chloride replaced with SCN^-. The same solution was used in the second period in expt. 7.

The membrane potential in NO_3-Ringer's fluid

The slowing of the efflux of ^{36}Cl in NO_3-Ringer's fluid might be due to a changed permeability to the chloride ion, or to a change in the membrane potential. The second possibility is excluded because the membrane potential in NO_3-Ringer's fluid does not differ from the potential in Cl-Ringer's fluid. The mean internal potential for one muscle in Cl-Ringer's fluid was $-90·5 \pm 0·9$ mV (16 fibres), and for the same muscle in NO_3-Ringer's fluid it was $-91·5 \pm 0·6$ mV (17 fibres). The mean internal potential of a second muscle was $-89·3 \pm 1·3$ mV (12 fibres) in NO_3-Ringer's fluid, and in Cl-Ringer's fluid before and after the measurement in nitrate it was $-90·4 \pm 1·3$ mV (11 fibres) and $-89·9 \pm 1·2$ mV (10 fibres) respectively ($\pm$ S.E. of mean). The size of the action potential was unaltered, but the negative after-potential was larger and of longer duration. These results confirm the observations of Etzensperger & Bretonneau (1956) and Hutter & Padsha (1959). No transient depolarization, which would be expected if the membrane was less permeable to nitrate than to chloride, was detected when the fluid flowing past an impaled fibre in a whole sartorius muscle was changed from Cl- to NO_3-Ringer's fluid. Hodgkin & Horowicz (1959), using isolated muscle fibres under similar conditions, found either no change in resting potential or a hyperpolarization. They have explained their results in terms of a decrease of chloride permeability in NO_3-Ringer's fluid.

The efflux of chloride from muscles with a raised internal chloride concentration

A small number of experiments was done to estimate the efflux of chloride into a Ringer's fluid to which an extra 47·5 m-mole KCl/l. had been added. Muscles were loaded with tracer by soaking for 2½ hr in a solution of this composition made up with $Na^{36}Cl$. They were then transferred to an inactive solution of the same composition, and the efflux was followed in the usual way. The losses of extracellular and intracellular chloride were easily separated, because in Ringer's fluid with extra KCl the fraction of the total muscle chloride which is intracellular is much greater than in Ringer's fluid. In a Ringer's fluid with KCl added to bring the external potassium concentration to 50 mM the internal chloride concentration at equilibrium is 47 m-mole/kg fibre water, and the muscle fibres should have reached equilibrium in 2½ hr soaking (Adrian, 1960). The mean time constant for the loss of ^{36}Cl was 31 ± 7 min (S.E. of mean 4 expts.). This corresponds to an efflux of 50 ± 11 pmole/cm² sec. Keynes's (1954) approximation for the effect of extracellular diffusion suggests that this figure is about 5% less than the true flux.

DISCUSSION

The internal chloride concentration of muscle fibres in Cl-Ringer's fluid (2·5 mM-K, 120 mM-Cl) lies between 3·1 and 3·8 m-mole/kg fibre water. Although the method of estimation necessitates a diffusion correction of rather uncertain magnitude, the value agrees well with the idea that the electrochemical potential of chloride is equal on the two sides of the membrane under these conditions. On this basis $[Cl_i]$ would be equal to $[Cl_o]$ exp (VF/RT), where V is the internal potential. For an internal potential of -92 mV and an external chloride concentration of 120 mM, the expected value of the internal chloride concentration is 3·1 mM. Table 3 shows the membrane potentials of muscle fibres which have been allowed to come into equilibrium with eight different solutions. For a particular value of the external potassium concentration the presence of chloride makes little or no difference to the membrane potential. If the distribution of chloride were passive this result would be expected, even for a membrane highly permeable to chloride. Direct estimations of the equilibrium internal chloride concentration in solutions with a raised potassium content are also consistent with prediction (Boyle & Conway, 1941; Adrian, 1960).

The experiments also show that the presence of nitrate and thiocyanate in the external solution affects the ease with which chloride can cross the muscle fibre membrane. Harris (1958) showed that the efflux of chloride

is slowed when external chloride is replaced by Br^-, NO_3^-, I^-, ClO_4^-, or CNS^-. Hutter & Padsha (1959) and Hodgkin & Horowicz (1959) have pointed out that the absence of a change in membrane potential when nitrate is suddenly substituted for chloride suggests that the effect of nitrate is to reduce the chloride permeability until it about equals the nitrate permeability.

Since the outward movement of chloride is not independent of the external anion, it is reasonable to ask whether the inward and outward

TABLE 3. Membrane potentials at equilibrium in solutions with and without chloride

$[C_o]$ (m-osmole/l.)	$[K_o]$ (mM)	$[Cl_o]$ (mM)	Internal potential (mV)
250	2·5	120	−92
250	2·5	0	−90*
250	10	120	−60
250	10	0	−61
250	50	120	−26
250	50	0	−25
450	100	220	−20
450	100	0	−20

Chloride replaced by sulphate; data previously published (Adrian, 1956, 1960). * Unpublished observations.

movements of chloride are independent. If the movements of chloride are independent the efflux may be calculated by the constant field theory:

$$M^o_{Cl} = P_{Cl} \frac{VF}{RT} \frac{[Cl_i]\exp(-VF/RT)}{1-\exp(-VF/RT)},$$

where V is the internal potential (Keynes, 1951). Hodgkin & Horowicz (1959) concluded that the chloride permeability (P_{Cl}) remains constant when either the membrane potential or the external chloride concentration are altered, and that its value is 4×10^{-6} cm/sec. The calculated value for the efflux in Cl-Ringer's fluid is 44 pmole/cm² sec. An efflux of this magnitude would result in a time constant for the loss of ^{36}Cl into Cl-Ringer's fluid of about $2\frac{1}{2}$ min. The loss of intracellular chloride would then be quite indistinguishable from the loss of extracellular chloride. In the present experiments the time constant in Cl-Ringer's fluid (11 min) could only be measured after the muscle fibres had lost chloride into NO_3-Ringer's fluid, during the subsequent period when the fibres were gaining chloride from the Cl-Ringer's fluid. If the inward chloride movement interfered with the outward movement, the time constant for the loss of ^{36}Cl into Cl-Ringer's fluid would be less after the muscle had been in NO_3-Ringer's fluid than when the muscle was in the steady state. If the influx and efflux were independent, the time constant for the steady state and the conditions of these experiments would be the same. A time

constant of 11 min would correspond to a flux of about 10 pmole/cm² sec and a membrane chloride conductance of 38 μmho/cm². Electrical measurements of the total conductance indicate a value of 250 μmho/cm² (Fatt & Katz, 1951), two thirds of which is thought to be chloride conductance (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). It is obvious that a flux of 10 pmole/cm² sec is too small to account for the chloride conductance of the membrane. The discrepancy could be explained by the type of mechanism suggested by Hodgkin & Keynes (1955) to account for the potassium flux ratios in *Sepia* axons poisoned with DNP.

In the chloride-containing solution with a potassium concentration of 50 mM, at equilibrium, the internal potential is -32 mV and the internal chloride concentration is 47 m-mole/kg fibre water (Adrian, 1960). Using the same value for P_{Cl} (4×10^{-6} cm/sec) as was used to calculate the flux in Cl-Ringer's fluid (2·5 mM-K), the calculated flux in the high potassium solution (50 mM-K) is 330 pmole/cm² sec. The observed flux is only 50 pmole/cm² sec. In both cases the difference could be accounted for if P_{Cl} was less than 4×10^{-6} cm/sec, but the large size of the discrepancy suggests that the movements of chloride are not independent.

SUMMARY

1. The intracellular chloride concentration of muscle in Ringer's fluid is consistent with a purely passive distribution of chloride. It is estimated to be between the limits of 3·1 and 3·8 m-mole/kg fibre water.

2. The presence of NO_3^- and SCN^- in the external fluid reduces the permeability of the membrane to chloride.

3. It is tentatively concluded that the chloride influx and efflux are not independent.

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REFERENCES

ADRIAN, R. H. (1956). The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* **133**, 631–658.

ADRIAN, R. H. (1960). Potassium chloride movement and the membrane potential of frog muscle. *J. Physiol.* **151**, 154–185.

BOYLE, P. J. & CONWAY, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1–63.

EDWARDS, C., HARRIS, E. J. & NISHIE, K. (1957). The exchange of frog muscle Na^+ and K^+ in the presence of the anions Br^-, NO_3^-, I^-, and CNS^-. *J. Physiol.* **135**, 560–566.

ETZENSPERGER, J. & BRETONNEAU, Y. (1956). Potentiel consécutif et durée de l'état actif de la fibre musculaire striée. Action des ions NO_3^-, Br^- et I^-. *C.R. Soc. Biol. Paris*, **150**, 1777–1781.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

HARRIS, E. J. (1958). Anion interaction in frog muscle. *J. Physiol.* **141**, 351–365.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium ions between the muscle and the surrounding medium. *Trans. Faraday Soc.* **45**, 508–528.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & KEYNES, R. D. (1955). The potassium permeability of a giant nerve fibre. *J. Physiol.* **128**, 61–88.

HUTTER, O. F. & NOBLE, D. (1960). The chloride conductance of frog skeletal muscle. *J. Physiol.* **151**, 89–102.

HUTTER, O. F. & PADSHA, S. M. (1959). The effect of nitrate and other ions on the membrane resistance of frog skeletal muscle. *J. Physiol.* **146**, 117–132.

KEYNES, R. D. (1951). The ionic movements during nervous activity. *J. Physiol.* **114**, 119–150.

KEYNES, R. D. (1954). The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B, **142**, 359–382.

LEVI, H. & USSING, H. H. (1948). The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. *Acta physiol. scand.* **16**, 232–249.

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